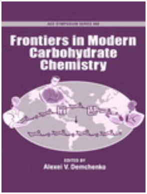

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Frontiers in Modern Carbohydrate Chemistry

EDITED BY
Alexei V. Demchenko

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ACS SYMPOSIUM SERIES **960**

Frontiers in Modern Carbohydrate Chemistry

Alexei V. Demchenko, Editor
University of Missouri–St. Louis

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American Chemical Society, Washington, DC

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Foreword

The ACS Symposium Series was first published in 1974 to provide a mechanism for publishing symposia quickly in book form. The purpose of the series is to publish timely, comprehensive books developed from ACS sponsored symposia based on current scientific research. Occasionally, books are developed from symposia sponsored by other organizations when the topic is of keen interest to the chemistry audience.

Before agreeing to publish a book, the proposed table of contents is reviewed for appropriate and comprehensive coverage and for interest to the audience. Some papers may be excluded to better focus the book; others may be added to provide comprehensiveness. When appropriate, overview or introductory chapters are added. Drafts of chapters are peer-reviewed prior to final acceptance or rejection, and manuscripts are prepared in camera-ready format.

As a rule, only original research papers and original review papers are included in the volumes. Verbatim reproductions of previously published papers are not accepted.

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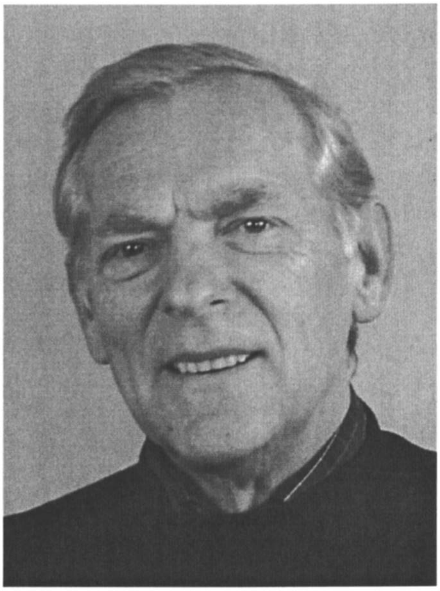
In Memorium



JacquesH. van Boom

(May 14, 1937–July 31, 2004)

Jacques H. van Boom received his Ph.D. thesis cum laude in 1968 at the University of Utrecht, under the guidance of Dr. Brandsma and Prof. Dr. Arends. He continued his training at the University of Cambridge, where he was a Ramsay Memorial Postdoctoral Fellow in the group of Lord Todd, from 1968 until 1970. He started his independent career at Leiden University (Leiden, The Netherlands) in 1970 as a lector and became holder of the chair in Bioorganic Chemistry in 1978. After his retirement, in June 2002, he remained actively involved in teaching and research as scientific advisor of the Faculty of Sciences at Leiden University. Jacques van Boom has supervised more than 60 Ph.D. students and numerous undergraduate students. He is the author of more than 750 scientific publications that collectively have received more than 25,000 citations. In 1981 he was elected as Fellow of the Royal Dutch Academy of Sciences (KNAW). He is the recipient of several prestigious scientific awards, including the Gold Medal of the Royal Dutch Chemical Society (KNCV, 1975), the Royal Shell Award (1985), The Simon Stevin Master Award (1999), and the Akzo Nobel Science Award (2000).

Throughout his scientific career, Jacques van Boom devoted his skills as a bioorganic chemist to contribute to our understanding of processes revolving around what he liked to call the *Central Dogma* in biology. Early on he realized the tremendous power of organic chemistry as a means to deepen our understanding of the complex biological interactions that are at the basis of life. As the processes within the central dogma—transcription, translation, protein synthesis, and metabolite processing—all involve various biopolymers, and their interaction, the basic aim in his research was to prepare fragments of these, and their synthetic analogues. In the selection of his research objectives, he always felt that, rather than pursuing the synthesis of complex molecules, the prime objective of the organic chemist should be to create compounds with unique properties that facilitate biophysical and biological research and that cannot be obtained otherwise. In general, he followed a three-pronged strategy. First, he would select a specific process to which he would apply his synthetic skills. For this, he assembled a wide international network of collaborators who were active in diverse biological disciplines. Second, he would determine the synthetic technology required for the preparation of the target compounds. Since, in many cases, such methodology was not known at that time, at least not for general implementation, he and his research team ended up developing new methodologies themselves and thus contributed in the discovery of important general synthetic strategies. Third, the target compounds would be prepared in useful quantities and with high purity and evaluated in the proper context, and in collaboration with the aforementioned partners.

Because the basic information of life is stored in DNA, Jacques van Boom started his independent research career with the pursuit of synthetic strategies toward DNA oligomers. Presently, DNA synthesis is a highly standardized, automated process, and oligomers of up to a 100 nucleotides can be ordered from many companies. At the time Jacques

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van Boom became active in the field, in the middle of the 1970s, the successful synthesis of even small DNA oligomers entailed a tremendous scientific challenge. Jacques van Boom maintained a pre-eminent position in the area of DNA synthesis by the development of a series of new phosphorylation techniques, the most prominent of which is his modification of the phosphotriester method. With this 'van Boom's phosphotriester modification' it was, for the first time, possible to synthesize DNA fragments both on a large scale and in an automated, solid support fashion. Indeed, this method has been the method of choice for a number of years, up until the phosphoramidite methodology came to the fore. The in-house strategy gave van Boom an important edge in the field, and he has provided the scientific community in the 1970s and early 1980s with literally hundreds of synthetic oligomers, with which many exciting discoveries have been made. One highlight, achieved in collaboration with Alex Rich (Massachusetts Institute of Technology, Boston, Massachusetts), is the first single atom structure of a defined DNA duplex and the discovery, based on this, of Z-DNA as a new type of duplex DNA. Another highlight is the elucidation of the mode of action of the DNA targeting antibiotic bleomycin, in collaboration with Sidney Hecht (University of Virginia, Charlottesville, Virginia).

In the early 1980s, at the pinnacle of his fame as a nucleic acid chemist, Jacques van Boom realized that for him to remain an important figure in bioorganic chemistry, a change from monodisciplinary research to multidisciplinary research was essential. He widened his horizon and tackled many synthetic challenges involving not onlythe preparation of oligomeric fragments of the other biopolymers, peptides, and carbohydrates but also hybrid structures thereof (nucleopeptides and glycosylated nucleotides) and compounds able to interfere with their biosynthetic assembly (enzyme inhibitors). Several highlights are the following.

In collaboration with the Dutch biochemist Piet Borst (Netherlands Cancer Institute, Amsterdam, The Netherlands), a program aimed at the evaluation of the role of a specific glycosylated nucleobase, denominated J (beta-glucosylated 5-hydroxymethyl-deoxyuridine), as present in the genome of the human pathogen *Tryptanosoma brucei* (the causative of the African sleeping sickness) was started. Synthetic J and oligonucleotides of which J is a structural element proved to be invaluable in the generation of specific polyclonal antibodies. With these, not only detection of the presence of small amounts of *T. brucei* is possible, but also the presence of the J modification in other human pathogens of the Kinetoplastida family was discovered.

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In collaboration with Eckard Wimmer (State University of New York, Stony Brook, New York) a breakthrough in our understanding of the replication mechanism of the poliovirus was achieved. Key to this study proved to be the accessibility of homogeneous uridylylated oligopeptide fragments. In itself, the chemical synthesis of peptide nucleic acid fragments of the complexity required for these studies, with the many synthetic hurdles that are caused by the incompatibility of standard peptide synthesis and nucleic acid synthesis protocols, represents a milestone in the oeuvre of Jacques van Boom and a hallmark in bioorganic chemistry.

An early highlight in the area of glycobiology comprises the development of a synthetic vaccine against Haemophilus influenzae type b (Hib), the causative of pneumonia and meningitis. Indeed, with this research executed in the end of the 1980s and beginning of the 1990s van Boom proved to be far ahead of his time. In collaboration with the National Institute for Public Health and the Environment (RIVM, Bilthoven, The Netherlands), synthetic strategies for the preparation of oligomers of the Hib capsular polysaccharide were developed. With conjugates of these, and the immunogenic response they elicit, infant monkeys were protected successfully against Hib infections. At that time, the strategy was abandoned, primarily because there was no interest from industrial partners (who did not believe in the economical viability of synthetic vaccines) for further development. However, very recently a highly similar strategy has led to a successful synthetic conjugate vaccine against Hib, which is now applied in Cuba (in fact the synthetic strategy towards the synthetic oligosaccharide is essentially that reported by van Boom).

Of more fundamental importance to the synthetic glycobiology community are the pioneering efforts in the assembly, on a solid support, of synthetic oligosaccharide fragments. The automated solid phase synthesis of oligosaccharides and glycoconjugates of any desired length and nature, with the ease now customary for oligonucleotide- and oligopeptide synthesis, is one of the holy grails in contemporary organic chemistry. In recent years, many research groups have made forays in this area. The first report that aims at automated oligosaccharide synthesis describes research by van Boom and co-workers on the use of polyethylene glycol as carrier in a solid-phase synthesis of a galactofuranose oligomer. This striking early example was carried out many years before other oligosaccharide synthesis groups dared to enter the arena of solid-phase assisted oligosaccharide assembly.

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Another hallmark with fundamental implications is the discovery, also in the early 1990s, of a set of activator systems that turn thioglycosides into effective donors for oligosaccharide assembly. At that time anomeric thioethers were regarded as suitable temporary protective groups because of their intrinsic stability towards many synthetic conditions. With the development of suitable thiophilic promoter systems, thioglycosides can now be employed as donor or acceptor in oligosaccharide synthesis, thereby markedly reducing the complexity of the synthesis of many oligosaccharides and glycoconjugates. The describing this advent is one of the most cited papers in modern carbohydrate chemistry and the thioglycoside methodology is one of the most widely explored and applied strategies for the assembly of oligosaccharides and glycoconjugates, both in solution and on the solid support.

Jacques van Boom reached his eminent position in bioorganic chemistry through a combination of utter dedication to research, complete confidence in his ability to recognize potential in his students and his remarkable intuition in selecting his research objectives. His devotion to research was well known in the Netherlands, where he was famous for being absent at institutional management meetings. To date, the term *van Booming* describes not showing up at specific meetings, and on the rare occasions he did appear his colleagues often felt that an important decision might be in the making. Jacques van Boom presided over both the intellectual and internal wellbeing of his pupils and did not shy away from menial tasks. He took it upon himself to distill most of the essential solvents that, apart from taking away this labor from his students, provided him with the necessary excuse to leave those board meetings he did get caught up in. Further, he presided over the preparation of coffee for the whole laboratory, and he regarded coffee making as a continuous extraction process, which required only the addition of some extra coffee beans at certain times.

Jacques van Boom supervised more than 60 Ph.D. students and numerous postdoctoral fellows, many of which have reached eminent positions, both within the Netherlands and abroad, and both in academia and in industry. Roberto Crea, a Postdoctoral Fellow in the mid 1970's, has had a major impact on the evolvement of Genentech as a leading Biotech company by the establishment of an automated DNA synthesis facility. Peter Burgers, an early Ph.D. student, became interested in molecular biology in later years and is now a professor at Washington University, St Louis, Missouri. Geert-Jan Boons, Ph.D. student in the mid 1980s, is a professor in glycobiology at Athens, Georgia. Stan van Boeckel, who defended his thesis in the beginning of the 1980s, continued his career at the leading Dutch Pharmaceutical Company, Organon, where he is now Head of Medicinal Chemistry. One highlight in his career is the development (together with Maurice Petitou) of the anti-coagulating agent Arixtra, a recently marketed synthetic heparin pentasaccharide. Jan de Rooij, another early Ph.D. student, ended his career as Head of Scientific Research of the Dutch-British food giant Unilever. Many other former students of Jacques van Boom found their way to the Dutch chemical and pharmaceutical industry, next to Organon and Unilever also Diosynth, Solvay Pharmaceuticals, Philips, Akzo Nobel and DSM. Still others have found employment at prominent Dutch research institutes, universities, and university hospitals, where they are active in many different research areas, not only in organic and bioorganic chemistry but also in homogeneous catalysis, inorganic chemistry, and biochemistry. Thus, Jacques van Boom has made a large impact on chemistry-based scientific research both in the Netherlands as well as abroad.

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Preface

Carbohydrates are the most abundant biomolecules on Earth, nevertheless, their chemistry and biology has been a *Cinderella* field. Our current knowledge about these fascinating natural compounds is not yet complete; however, thanks to the explosive growth of the field of glycobiology in the recent years, we already know that this Cinderella is working extremely hard in our bodies, although in the shadow of her *sisters,* genomes and proteins. Indeed, carbohydrates are involved in a broad range of vital biological processes, such as fertilization, antiinflammation, immunoresponse, joint lubrication, and antigenic determination. Carbohydrates are also responsible for many damaging processes in our cells, such as bacterial and viral infections, development and growth of tumors, metastasis, and tissue rejection. Many of these processes are directly associated with various deadly diseases of the 21st century: AIDS, cancer, meningitis, hepatitis, septic shock, to name a few. Elucidation of the exact mechanisms of the carbohydrate involvement in disease progression would be significantly facilitated if we could rely on the comprehensive knowledge of the structure, conformation, and properties of the carbohydrate molecules.

Although scientists learned to selectively cleave, isolate, purify, and characterize certain classes of naturally occurring glycostructures, their accessibility in pure form is still quite limited. In this case, a chemical or chemoenzymatic approach can provide an access to significantly larger quantities of chirally pure material. Even on the laboratory scale, chemical synthesis of complex carbohydrates is regarded as laborious and inefficient; in industry, things only get worse as a number of additional factors and considerations become important. Pharmaceutical and biotechnological companies have already demonstrated an interest in producing complex oligosaccharides or glycoconjugates; however, these examples are scarce and far apart. It is critical to make complex carbohydrates more accessible to general chemical, biochemical, and industrial audience to keep pace with the exploding

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area of glycosciences. This can be only achieved by the development of general methods for stereocontrolled glycoside synthesis and reliable strategies for convergent oligosaccharide assembly that would be applicable to both laboratory and industrial preparation.

The Symposium on *Frontiers in the Modern Carbohydrate Chemistry* was organized as a part of the program of American Chemical Society Division of Carbohydrate Chemistry within the 229th American Chemical Society (ACS) National Meeting (March 13–17, 2005, San Diego, California). The scientific program of the Symposium encompassed 12 plenary lectures. The Symposium discussed many novel aspects of Glycosciences of the 21st century including such topics as new synthetic methodologies, diversity and target-oriented synthesis of complex glycostructures, medicinal chemistry, drug discovery, and biology of carbohydrates.

The main theme of the Symposium, modern carbohydrate chemistry, has become the base for this Symposium Series book. The scope of the book has been further expanded by inviting a number of renowned carbohydrate chemists, which resulted in 20 contributions. This Symposium was dedicated to the memory of Professor Jacques van Boom who was undoubtedly among the most influential figures of the carbohydrate chemistry renaissance of the 1980's and 1990s. The memoriam in this book is presented by former van Boom's associates - Herman Overkleeft, Mark Overhand, and Gijs van der Marel.

The editor has identified five significant categories, in which the contributions have been conservatively classified. Many scientists are working in the emerging areas, and sometimes it was very difficult to unambiguously classify one's work into a single category.

The section Synthesis of Modified Monosaccharides as Biological Probes and Therapeutic Agents describes the synthesis of relatively simple modified monosaccharides as biological probes and therapeutic agents, herein the following contributions have been presented. Paulson and co-workers describe the synthesis of substituted sialic acids, their incorporation into cell surface glycoproteins of B cells via the normal cellular biosynthetic pathway, and their use as probes for CD22–ligand interactions. It has been demonstrated that the substituted sialic acid analogs provide unique environment for the study of CD22–ligand interaction. Wang, Sun, and co-workers report their study of indolocarbazoles and anthracyclines as two important classes of glycosylated anticancer drugs. The authors obtained insightful information through

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the structure–activity relationship studies of these compounds by introducing uncommon deoxy and amino sugars. Field, Nepogodiev, and Jones report continuation of the studies related to chemical synthesis of plant cell wall glycan fragments. Herein, the authors describe an effective synthesis of the novel branched sugar aceric acid (Ace*f*), a unique component of rhamnogalacturonan, and its C-2 epimer. Marzabadi and DeCastrol developed a new one-pot method for the synthesis of 2-deoxy-*N*-glycosylamides by iodoamidation of 1,2-dehydro derivatives. This method offers a relatively simple entry into important classes of natural glycoconjugates, N-linked glycoproteins and glycopeptides, compounds of a tremendous biological significance, and therapeutic potential.

The nest section New Methods for Stereoselective *O*-Glycosylation describes some new methods for stereoselective glycosidic bond formation, herein the following contributions have been presented. Crich presents stereocontrolled synthesis of β-D-rhamnopyranoisdes from mannosyl donors bearing a modified 4,6-*O*-benzylidene group that functions as a stereodirecting precursor to the rhamnoside. In addition, synthesis of an alternating β-(1→3)/β-(1→4)-mannan and the development of propargyl moiety for improved stereoselectivity in β-mannosylations are discussed. Boons and co-workers describe an elegant novel approach to stereoselective α-glycosylations whereby a chiral auxiliary at C-2 of a glycosyl donor controls the anomeric outcome of a glycosylation. Fraser-Reid et al. discuss the influence of protecting groups on stereo-, chemo-, and regioselectivity of glycosidations. It has been determined that the preferential glycosylation of primary versus secondary hydroxyl holds only for disarmed donors, whereas armed donors are frequently secondary-OH selective. De Meo discusses the influence of an N-substituent at C-5 on the outcome of chemical sialylations. Recent scientific discovery of a strong effect of the C-5 amino substituent on the reactivity of both sialyl donors and acceptors has opened new perspectives for the field of chemical sialylation. Recent achievements in this area have been classified and overviewed.

The section New Strategies for Convergent Oligosaccharide Synthesis describes various strategic approaches to convergent oligosaccharide synthesis, herein the following contributions have been presented. Kim and Jeon discuss application of novel 2′-carboxybenzyl glycosyl donors to the efficient construction of oligosaccharides by the active-latent glycosylation strategy. Seeberger and Codée describe the

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synthesis of glycosyl phosphates and their use in the construction of complex oligosaccharides, both in solution and on solid support. Easy accessibility and rapid activation of glycosyl phosphates are important features for their successful application in automated solid-phase oligosaccharide synthesis. Demchenko and co-workers discuss the synthesis and application of glycosyl thioimidates as glycosyl donors in stereoselective glycosylation and convergent oligosaccharide assembly. This overview is primarily focused on the *S*-benzoxazolyl (SBox) and *S*-thiazolinyl (STaz) glycosyl donors, the unique activation conditions of which allowed the design of conceptually novel strategies for oligosaccharide synthesis. Such innovative approaches as the inverse armed–disarmed strategy, the *O*-2/*O*-5 cooperative effect in glycosylation, the temporary deactivation concept, and orthogonal activation of the STaz vs. *S*-ethyl moieties are discussed. Van der Marel et al. present an overview on oligosaccharide synthesis with a focus on chemoselective glycosylations. Thioglycosides are versatile building blocks and their application to oligosaccharide assembly has been further enhanced with the development of the sulfonium ion activator systems. Schmidt et al. discuss novel aspects of the solid-phase oligosaccharide synthesis. The authors designed the hydroxymethylbenzyl benzoate spacer-linker system connected to the Merrifield resin, which worked very well with glycosyl trichloroacetimidates as powerful glycosylating agents. The synthesis of a variety of oligosaccharides including a small library of complex-type N-glycans was accomplished in high overall yields and high purity.

The section Chemical and Enzymatic Synthesis of Therapeutically Significant Complex Glycostructures describes target syntheses of complex oligosaccharides and glycoconjugates by chemical and/or enzymatic techniques, herein the following contributions have been presented. Pozsgay and Kubler-Kielb describe the synthesis of oligosaccharide antigens related to the repeating units of the O-specific polysaccharide of *Shigella dysenteriae* type 1, a major cause of dysentery. The synthetic material is then covalently attached to the protein carrier, in order to obtain a well defined synthetic glycoconjugate vaccine. Linhardt and co-workers overview the chemoenzymatic synthesis of glycosaminoglycan analogs with the use of three families of enzymes including the polysaccharide lyase enzymes, bacterial enzymes, and glycosaminoglycan sulfotransferases that mediate important biosynthetic processes. Nikolaev and co-workers describe the chemical synthesis of glycosylphosphatidylinositol anchors from *Trypanosoma cruzi,* a causative agent of Chagas' disease. Promising proinflammatory activity of the natural fraction of *T. cruzi* trypomastigote mucins has stimulated the authors to pursue the development of efficient synthetic pathways for the chemical synthesis of structurally related glycoconjugates. Chang and Wang describe a glycodiversification concept and its application to the synthesis of Neomycin and Kanamycin aminoglycoside antibiotics and their analogs active against infectious diseases. The prevalence of the resistant bacteria has limited aminoglycoside usage and called for novel structurally modified derivatives, many of which showed promising activity against both susceptible and resistant strains of *Escherichia coli.* Michael highlights modern convergent approaches to synthesizing N-glycopeptides. While this class of natural compounds can be synthesized convergently by condensation of glycosamines with aspartic acid moiety of peptides, side reactions of aspartimide formation, hydrolysis, or peptide rearrangement may significantly reduce the overall efficiency of the conjugation. This challenge necessitates orthogonal protection of peptides, which is thoroughly discussed therein.

The section Special Topics of Modern Glycochemistry presents special topics of modern glycochemistry, herein the following contributions have been presented. Belitsky and Stoddart describe the development of self-assembled multivalent pseudopolyrotaxanes as flexible and dynamic neoglycoconjugates for binding Galectin-1. It has been determined that pseudopolyrotaxanes comprised of lactoside-displaying cyclodextrin "beads" threaded onto polyviologen "strings" efficiently precipitated Gal-1 and provided valency-corrected enhancements of up to 30-fold over native lactose. Barchi and Svarovsky report the preparation of quantum dots coated with the tumor-associated carbohydrate antigen βGal(1→3) αGalNAc and the investigation of their photoluminescent and binding properties. These new tools can be applied to labeling cells that express specific carbohydrate-binding proteins.

I express my sincere gratitude to the administrative personnel of the Division of Carbohydrate Chemistry for their help in organizing the Symposium; the ACS Books Department for their cooperation throughout the book preparation and publication process; and to all scientific contributors, discussion leaders, and reviewers for their continuous support and encouragement. A special coin of appreciation

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Symposium Outline (Sunday, March 13, 2005)

MORNING Session 1: H. Overkleeft, Presiding
8:30 — Introductory Remarks. A. V. Demchenko, Organizer
8:40 — 1. Paulsen's donor/acceptor "match" and selectivities in oligosaccharide synthesis.
B. Fraser-Reid, A. M. Gomez, J. C. López
9:10 — 2. Uncommon sugars and their conjugates to natural products.
P. G. Wang
9:40 — 3. New aspects of recent work on glycoconjugate synthesis.
R. R. Schmidt

MORNING Session 2: C. De Meo, Presiding
10:30 — 4. Stereoselective glycosylations using chiral auxiliaries.
G -J. Boons, J -H. Kim, H. Yang
11:00 — 5. Carbohydrate nanoscience: A new realm for biochemical and therapeutic applications. J. J. Barchi Jr.
11:30 — 6. Chemical tools for glycobiology.
C. R. Bertozzi

AFTERNOON Session 3: T. Lowary, Presiding
1:30 — 7. Sequential glycosylation strategies: A focus on thioglycosides as donors and acceptors.
G. A. van der Marel
2:00 — 8. Thioimidate approach to saccharide synthesis.
A. V. Demchenko
2:30 — 9. Advanced technologies applied to glycosyltransferase development.
J. Gervay-Hague, L. Ying, J. H. Wong

AFTERNOON Session 4: Z. Witczak, Presiding
3:20 — 10. Sialoside probes of Siglec-ligand interactions.
S. Han, B. E. Collins, T. Islam, P. Bengtson, N. Bovin, O. Blixt, J. Paulson
3:50 — 11. Chemical approaches to bacterial carbohydrate antigens.
V. Pozsgay, J. Kubler-Kielb, A. Fekete, P. Hoogerhout, B. Coxon
4:20 — 12. Stereocontrolled glycosylation: Recent advances.
D. Crich
4:50 — Concluding Remarks. A. V. Demchenko, Organizer

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Chapter 1

Synthesis of 9-Substituted Sialic Acids as Probes for CD22–Ligand Interactions on B Cells

Shoufa Han, Brian E. Collins, and James C. Paulson*

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As a member of the sialic acid binding immunoglobulin-like lectins (siglecs) family, the B cell protein CD22 binds to NeuAcα2-6Gal terminated glycans of glycoproteins on the same cell (in *cis*) and on adjacent cells (in *trans*). As a route to develop ligands with altered biological properties, sialic acid analogs with C-9 substitutions were efficiently synthesized from 9-azido-NeuAc, which was obtained in two steps in 65% overall yield from neuraminic acid. 9-Substituted sialic acid analogs were incorporated into cell surface glycoproteins of B cells via the normal cellular biosynthetic pathway, providing unique approaches for the study of CD22-ligand interaction, including *in situ* photoaffinity crossliking to *cis* ligands, and modulating the binding affinity with both *cis* and *trans* ligands in the cellular context.

In Frontiers in Modern Carbohydrate Chemistry; Demchenko, A.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2007.

Introduction

The sialic acids, a family of 2-keto-3-deoxynonulsonic acids that typically reside in the non-reducing termini of glycans, including glycolipids and glycoproteins, are widespread from bacteria to mammals. They are involved in host-pathogen interactions, cell-cell adhesion, and cell signaling events, all mediated by microbial and mammalian sialic acid binding proteins.[1-3] Although a very diverse family, the most common sialic acid species is 5-acetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-2-nonulosonic acid (N-acetyl-neuraminic acid, NeuAc). NeuAc modified with substituents at the 5- or 9-positions have demonstrated utility for developing useful tools to study the biological roles of sialylated glycans and developing inhibitors of the NeuAc-binding proteins.[4-8] NeuAc analogs modified with bio-orthogonal groups are also readily taken up by living cells and incorporated into glycoproteins, allowing subsequent selective chemical manipulations on cell surface for structure-function studies of these glycoproteins.[9-11] Recently, sialic acid substituted with a 4-biphenylcarbonyl (BPC) group at C-9 was demonstrated to be a potent inhibitor of CD22, providing renewed interest in exploiting 9-substituted NeuAc analogs as probes of the biology of sialic acid binding proteins.[7, 8]

CD22 is a negative regulator of B cell activation, mediated by recruitment of SH2 domain-containing phosphatase 1 (SHP-1) to the B cell receptor (BCR) through the cytoplasmic domain of CD22.[12] Activation of B cells by a foreign antigen results in proliferation, differentiation and production of antigen specific antibodies, representing a primary response of the immune system to pathogens. Hyper-activation of B cells, however, can also lead to pathologies such as autoimmune disease, illustrating a critical need for regulation of the immune response. Indicative of its role as a regulator of B cell activation, CD22 null mice exhibit a hyperimmune B cell phenotype and hallmarks of autoimmune disease.[13-17] As a member of the family of sialic acid-binding immunoglobulin-like lectins (siglecs), CD22 contains an extracellular N-terminal Ig domain that binds to the terminal sequence of N-acetyl neuraminic acid α2-6 galactose (NeuAcα2-6Gal), which is highly expressed on the carbohydrate groups of B and T cell glycoproteins.[18] Cell surface glycoproteins bind to CD22 both in *cis* (same cell) and in *trans* (adjacent cell; Figure 1). The *cis* binding 'masks' the ligand binding domain,[19] [20]modulating its activity as a regulator of B cell signaling.[7, 21, 22]

In our studies to elucidate CD22 *cis* ligands, NeuAc analogs with C-9 substituted *p*-azido-phenyl-acetamido- (AAz) or BPC group were synthesized and incorporated into cell surface glycoproteins. The enhanced CD22-ligand binding affinity and the functionality of the 9-AAz moiety was used for photoaffinity crosslinking glycans of the *cis* ligand to CD22 *in situ*.

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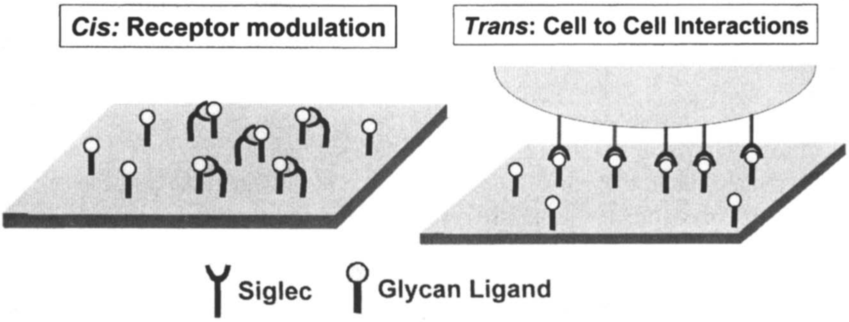

Figure 1. CD22 can interact with ligands both cis and trans. (Adapted with permission from reference 20. Copyright 2004 Elsevier) (See page 1 of color inserts.)

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Synthesis of 5-acetamido-9-azido-3,5,9-trideoxy-D-*glycero*-D-*galacto*-2-nonulosonic acid (9-azido-NeuAc, 3)

One of the general methods to introduce substitutions at C-9 involves amidation of 9-amino-NeuAc, which is obtained by reduction of 9-azido-NeuAc.[5,6] The routine use of 9-azido-NeuAc as a key intermediate in the synthesis of 9-substituted sialic acids is limited by lengthy procedures for its preparation. Current chemoenzymatic[23, 24] or chemical synthesis[6] methods for preparation of 9-azido-NeuAc involve multi-step, low yield procedures starting from either N-acetyl-mannosamine or NeuAc. We developed an efficient, high yield, two step synthesis of 9-azido-NeuAc as depicted in Scheme 1 and briefly described below. Detailed procedures can be found in the supplemental methods to Han *et al*[25] at http://www.nature.com/nchembio/journal/v1/n2/extref/nchembio713-S7.pdf.

NeuAc methyl ester was prepared in quantitative yield by stirring NeuAc (**1**) in dry methanol with a catalytic amount of TFA.[26, 27] Upon complete removal of methanol, treatment of NeuAc methyl ester with 1.05 equivalent of *p*-toluenesulfonyl chloride in pyridine at 0 °C provides 5-acetamido-9-tosyl-3,5,9-trideoxy-D-*glycero*-D-*galacto*-2-nonulosonic acid (**2**) in 76% yield. Complete conversion of **2** to 9-azido-NeuAc (**3**) was achieved by reaction with sodium azide in refluxing aqueous acetone, through simultaneous incorporation of the 9-azide and hydrolysis of the methyl ester (Scheme 1) in quantitative yield. Purification by column chromatography gives the pure compound in 83% yield.

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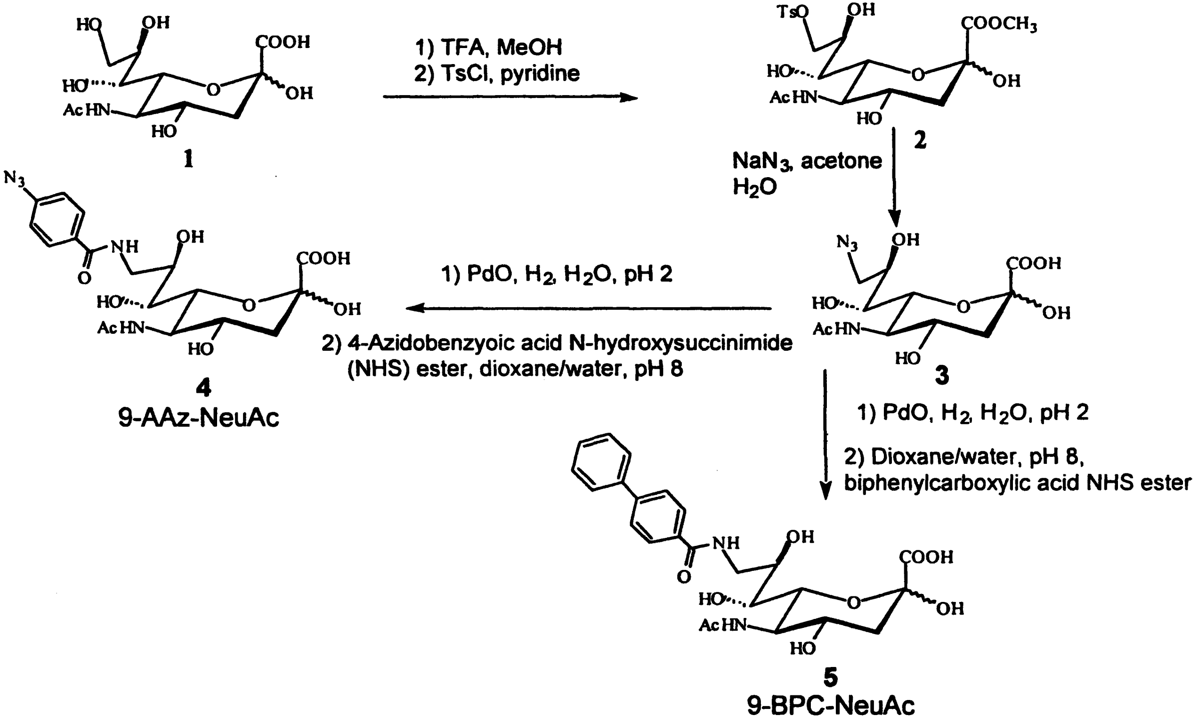
1
1) TFA, MeOH
2) TsCl, pyridine
2
NaN₃, acetone
H₂O
3
1) PdO, H₂, H₂O, pH 2
2) 4-Azidobenzyoic acid N-hydroxysuccinimide (NHS) ester, dioxane/water, pH 8
4
9-AAz-NeuAc
1) PdO, H₂, H₂O, pH 2
2) Dioxane/water, pH 8, biphenylcarboxylic acid NHS ester
5
9-BPC-NeuAc

Scheme 1. Synthesis of 9-substituted NeuAc analogs.

The fortuitous hydrolysis of the ester of **2** in the refluxing aqueous acetone was somewhat surprising since a previous report of the reaction employing the related α-methyl glycoside of **2** (methyl-α-(methyl-5-acetamido-9-tosyl-3,5-dideoxy-D-*glycero*-D-*galacto*-2-nonulopyranosid)onate (**6**), resulted in the retention of the methyl ester (Scheme 2).[6] To confirm the different stability of the methyl ester of the two compounds, **6** was synthesized and heated under reflux with sodium azide in aqueous acetone under identical conditions to that used for **2**. As previously noted,[6] no hydrolysis of the methyl ester was observed. Given the different reaction patterns of **2** and **6**, the existence of free C-2 hydroxyl group of **2** facilitates the hydrolysis.

Previous chemical methods for production of 9-azido-NeuAc from NeuAc involve synthesis of **6** used for introduction of 9-azide, followed by base hydrolysis of the methyl ester and acid hydrolysis of the methyl glycoside.[6] In the procedure reported here, no protection of the anomeric center is required, and the simultaneous hydrolysis of the methyl ester of **2** during introduction of the azide replaces the additional hydrolysis steps required for removal of the methyl groups of **6**. Since 9-azido-NeuAc (**3**) is readily converted into CMP-9-azido-NeuAc, a donor substrate for sialyltransferases, 9-azido-NeuAc also offers a convenient route for chemo-enzymatic synthesis of 9-substituted-NeuAc analogs of the more complex oligosaccharides recognized with high affinity by sialic acid binding proteins.[28, 29]

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Synthesis of 5-acetamido-9-(*p*-azidobenzoylamino)-3,5,9-trideoxy-D-*glycero*-D-*galacto*-2-nonulosonic acid (4) and 5-acetamido-9-(biphenylcarbonylamino)-3,5,9-trideoxy-D-*glycero*-D-*galacto*-2-nonulosonic acid (5)

Reduction of 9-azido-NeuAc (**3**) with H_2/PdO in acidic aqueous methanol affords 9-amino-NeuAc quantitatively. Acylation of 9-amino-NeuAc with NHS esters of various carboxylic acids at basic conditions (pH 8-9) readily affords various 9-substituted NeuAc derivatives in the yields ranging from 45% to 25%. To the solution of **3** in aqueous methanol, PdO was added and stirred under H_2 atmosphere with pH maintained at 1-2 by addition of acetic acid. The reaction mixture was filtered to remove the catalyst when it was complete as determined by TLC. To the fitrate was added dioxane, and 4-azidobenzoic acid NHS ester with pH maintained between 8.0-9.0 with saturated sodium bicarbonate solution. The mixture was stirred in the dark for 48 hr. The solvent was evaporated and the residue was purified by flash chromatography to give **4** in 45% yield. R_f:

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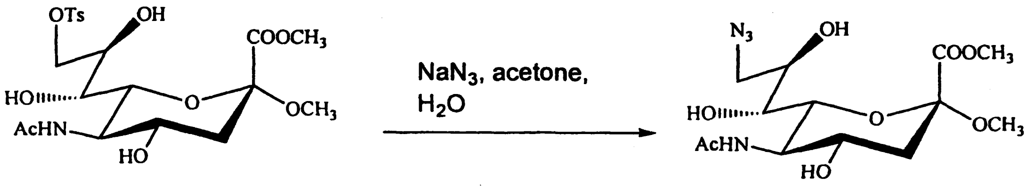
OTs
OH
COOCH$_3$
HO
O
OCH$_3$
AcHN
HO
NaN$_3$, acetone,
H$_2$O
N$_3$
OH
COOCH$_3$
HO
O
OCH$_3$
AcHN
HO

6 **7**

*Scheme 2. Conversion of **6** to α-methyl-9-azido-NeuAc methyl ester (7).*

0.70 (6:3:3:2 EtOAc:HOAc:MeOH:H_2O). Compound **5** was prepared using the same procedure using NHS ester of biphenylcarboxylic acid as the reagent in 25% yield. R_f: 0.50 (4:3:2 *i*-PrOH:MeCN:MeOH).

Incorporation of 9-substituted sialic acid into cell surface glycans

NeuAc modified with bio-orthogonal groups at the 5- or 9-positions are readily taken up by living cells and incorporated into glycoproteins via the normal biosynthetic pathway, allowing subsequent selective chemical manipulations on cell surface or structure-function studies of these glycoproteins.[9, 11, 30] Analogs of α-methyl-NeuAc with a phenyl or a biphenyl group at C-9 have been demonstrated to bind to CD22 with increased affinity.[7, 8] Accordingly, incorporation of NeuAc substituted with either an aryl azide (AAz) group or a biphenyl group at C9 into cell surface glycoproteins was anticipated to yield engineered carbohydrate ligands of 9-AAz/BPC-NeuAcα2-6Gal that would be recognized by CD22 (Figure 2). To accomplish this we used the B cell line BJAB K20 (K20), since it is deficient in UDP-GlcNAc-2-epimerase, the rate-limiting step in sialic acid biosynthesis.[31] Consequently, these cells are deficient in sialic acid, unless the media is supplemented with NeuAc, ManNAc or substituted NeuAc derivatives to initiate *de novo* synthesis of sialylated glycoproteins. Incubation of B cells with medium supplemented with 2 mM of 9-AAz-NeuAc or 9-BPC-NeuAc efficiently afforded corresponding cell surface glycoproteins with corresponding unnatural sialic acids. Formation of the cell surface 9-AAz-NeuAcα2-6Gal or 9-BPC-NeuAcα2-6Gal structure was verified by staining with FITC-labeled *Sambuccus nigra agglutinin* (SNA) that is specific for NeuAcα2-6Gal linkage as monitored by flow cytometry.[25] In the case of 9-AAz-NeuAc, the presence of azide functionality on the cell surface was verified by Staudinger-Bertozzi ligation, which resulted in covalent attachment of a biotin group to the cell surface.[25]

Incorporation of 9-AAz-NeuAc into cell surface glycans allows *in situ* photo-affinity crosslinking of CD22 to *cis* glycoprotein ligands

Cellular processing of 9-AAz-NeuAc affords cell surface glycoproteins with 9-AAz-NeuAcα2-6Gal which was indeed recognized by CD22 as determined by flow cytometry using a fluorescently labeled CD22-Fc chimera as a probe.[25] To determine if CD22 could be crosslinked to *cis* glycoprotein

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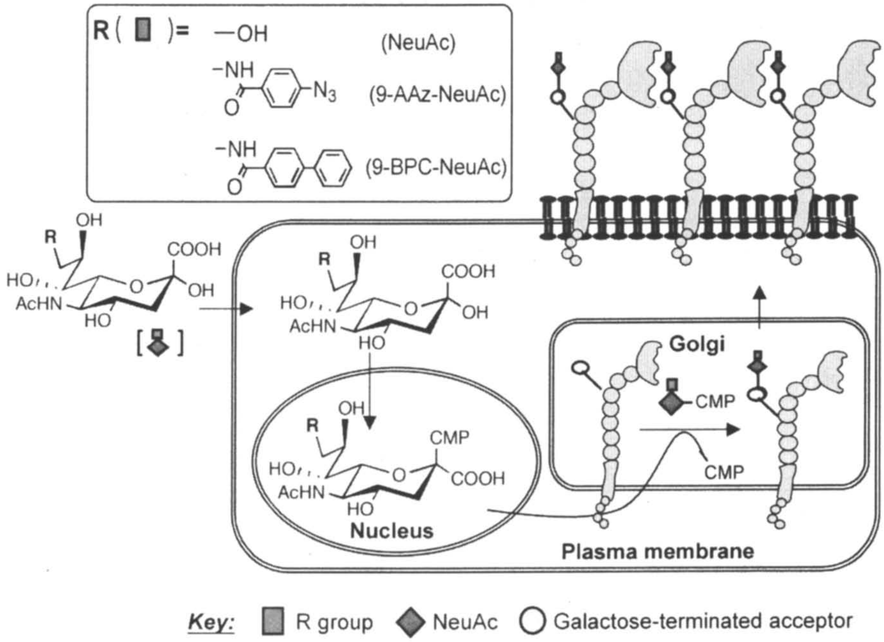

Figure 2. Strategy for introducing 9-substituted NeuAc into cell surface glycoprotein ligands of CD22. Modified sialic acids can be taken up by cells, converted to CMP-sialic acid, and transferred to nascent glycoproteins by sialyltransferases in the Golgi apparatus, and transported to cell surface. (Adapted with permission from reference 25. Copyright 2005 Nature Publishing.) (See page 1 of color inserts.)

ligands *in situ*, B cells cultured with NeuAc or 9-AAz-NeuAc were irradiated with UV light and the lysate was immunoprecipitated (IP) with anti-CD22 to isolate the CD22-*cis* ligand complexes. As illustrated in Figure 3, analysis by Western blotting showed that CD22 was extensively crosslinked. Subsequent analysis of the complexes[25] revealed negligible crosslinking to glycoproteins previously implicated as *cis* ligands, including CD45[32-34] and CD19.[35] Thus, despite the fact that these glycoproteins carry glycan ligands on the same B cell surface and their ligands can be recognized by CD22 *in vitro*, none of them appear to represent significant *cis* ligands of CD22 in resting B cells *in situ.*[25] However, direct evidence for crosslinking of CD22 to neighboring CD22 molecules was obtained, suggesting that CD22 forms homo-multimeric complexes in B cells facilitated by protein-glycan interactions.[25]

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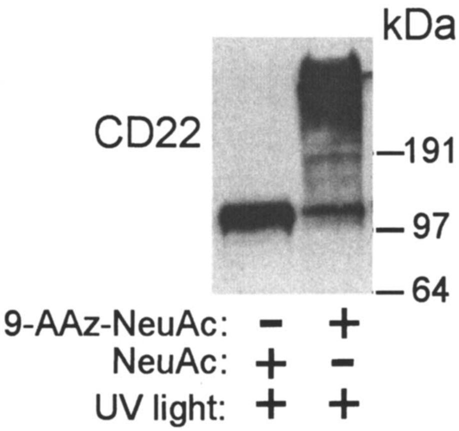

Figure 3. In situ UV cross-linking of CD22 and cis ligand in B cells.

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Incorporation of 9-BPC-NeuAc into cell surface glycans modulates CD22-ligand binding affinity *in situ*

The α-methyl glycoside of 9-BPC-NeuAc, is a 200 fold more potent inhibitor of CD22 relative to the α-methyl glycoside of NeuAc.[7, 8] We hypothesized that *de novo* synthesized cell surface CD22 sialoside ligand with a C-9 BPC group will have a higher binding affinity with CD22. This would allow investigation of the effect modulation the CD22-ligands affinity on CD22 function in B cells. 9-BPC-NeuAc was incorporated into cell surface glycans by culturing B cells with 9-BPC-NeuAc. On BPC-NeuAc cultured B cells, CD22 binds with both *cis* and *trans* ligands with enhanced affinity relative to B cells cultured with NeuAc. For example, as seen in Figure 4, B cells with cell surface 9-BPC-NeuAc aggregated heavily, indicative of CD22 interactions with *trans* ligands on adjacent cells.[36] Furthermore the *cis*-ligand-CD22 binding affinity is also greatly enhanced as BPC-NeuAc fed cells exhibit increased 'masking' of CD22 binding to sialoside probes (not shown).

Conclusions

9-Azido-NeuAc (**3**) was synthesized from commercial NeuAc in an efficient two-step procedure in 65% overall yield. 9-Azido-NeuAc (**3**) was easily derivatized to afford 9-substituted sialic acid analogs that are of great

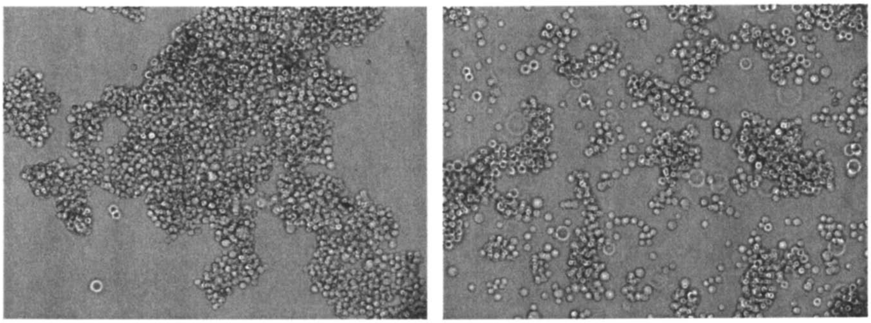

Figure 4. Increased aggregation of B cells cultured with BPC-NeuAc (2mM, left) as compared to NeuAc (2mM, right).

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value in the study of CD22 in B cells. With 9-AAz-NeuAc, we have demonstrated an approach to 'fish' for low affinity glycan ligand interactions that occur *in situ* by metabolic labeling of the glycan ligand with a photo-crosslinker. This approach is likely to have broader applicability to questions in biology relating to cell surface recognition of sialic acids,[3] and may similarly be extended to lectin interactions with other classes of glycans. In the case of 9-BPC-NeuAc, the binding affinity of CD22 and its ligands was greatly enhanced *in situ*, affording a direct approach to study the effect of ligand affinity on CD22 regulation of B cell activity. These experiments demonstrated the utility of 9-substituted sialic acid analogs that are of unique application in the study of sialic acid binding proteins.

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Chapter 2

Uncommon Sugars and Their Conjugates to Natural Products

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Indolocarbazoles (such as rebeccamycin, J-107088 and NSC 655649) and anthracyclines (such as doxorubicin and daunorubicin) are two important classes of anticancer drugs. Both natural and synthetic indolocarbazoles and anthracyclines contain mono-, or di-saccharides, which are critical parts of the molecules for binding to DNA or for inhibiting topoisomerases. Moreover, both natural and synthetic analogs containing disaccharides exhibit higher antitumor efficacy with unique DNA binding and topoisomerase poisoning characteristics. Thus, varying the uncommon sugar structure of indolocarbazole and anthracycline drugs will provide insightful information on the Structure-Activity Relationship (SAR) of topoisomerase I & II poisoning and DNA binding, and will potentially produce analogs for overcoming drug resistance in cancer therapy.

In Frontiers in Modern Carbohydrate Chemistry; Demchenko, A.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2007.

Numerous lead compounds in modern drug discovery are directly derived from natural products, many of which are glycosylated metabolites. Uncommon sugars, which are derived from common sugars by replacement of at least one hydroxyl group or hydrogen atom with another functional group, are frequently found in the secondary metabolites of microorganisms and plants, such as cardioglycosides, antibiotics, and anticancer agents *(1,2)*. The sugar moieties of these pharmaceutically important metabolites often play critical roles in determining the biological and pharmacological activities. Thus, modification or alteration of sugar structures has been validated as a powerful strategy in modern drug discovery *(3-5)*.

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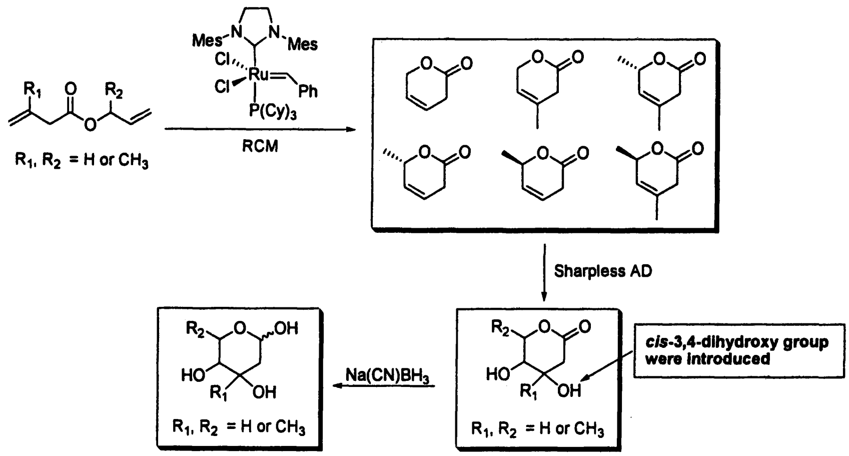
Uncommon Sugar Library

Deoxysugars and their oligosaccharides are frequently found in the structure of bioactive drugs. These uncommon sugars play very important roles in maintaining some drugs' activity. There have been continuing efforts on the synthesis of uncommon monosaccharides, starting from either carbohydrates or noncarbohydrate precursors *(6)*.

Systematic syntheses of uncommon sugars

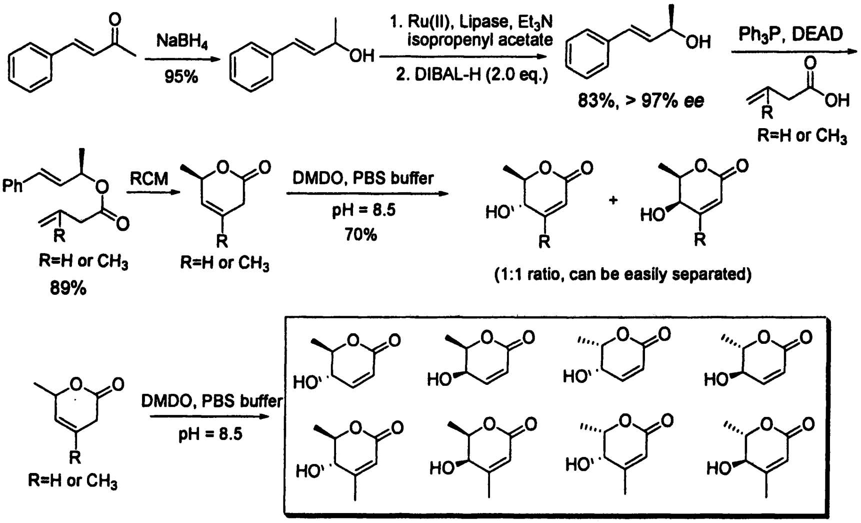
Traditionally uncommon sugars were synthesized through multistep transformations of relatively inexpensive common sugars *(7)*. One major disadvantage of this approach is the long reaction sequence for protection and deprotection manipulations. An alternative approach is using noncarbohydrate precursors. In this case, many chemists have come up with different synthetic approaches *(8-11)*. In 2002, our group developed a strategy for generating six-member β,γ-unsaturated lactones using Ring-Closing-Metathesis (RCM) *(12)*, as shown in Scheme 1. The major advantage of this synthetic strategy is that it provides a systematic synthesis pathway for producing desired uncommon sugar units.

In addition, we developed an alternative synthetic pathway by using an inexpensive starting material, *trans*-4-phenyl-3-buten-2-one *(13)*. The key intermediates for the uncommon sugars were achieved by this chemo-enzymatic synthetic pathway (Scheme 2). By using this method, four different types of deoxysugars were successfully prepared from one α, β-unsaturated lactone in moderate to good yields (Scheme 3). The major advantage of this method is that it allows the formation of uncommon sugars with both 3,4-*cis*- and 3,4-*trans*-difunctionalities.



R1 O R2
R1, R2 = H or CH3
Mes N N Mes
Cl Ru Ph
Cl P(Cy)3
RCM
Sharpless AD
cis-3,4-dihydroxy group were introduced
Na(CN)BH3
HO R1 OH
R1, R2 = H or CH3

Scheme 1. Synthesis of 2,6-dideoxysugar by RCM.



NaBH4
95%
OH
1. Ru(II), Lipase, Et3N isopropenyl acetate
2. DIBAL-H (2.0 eq.)
83%, > 97% ee
Ph3P, DEAD
R=H or CH3
RCM
89%
DMDO, PBS buffer
pH = 8.5
70%
(1:1 ratio, can be easily separated)
DMDO, PBS buffer
pH = 8.5

Scheme 2. Key intermediates for deoxysugars.

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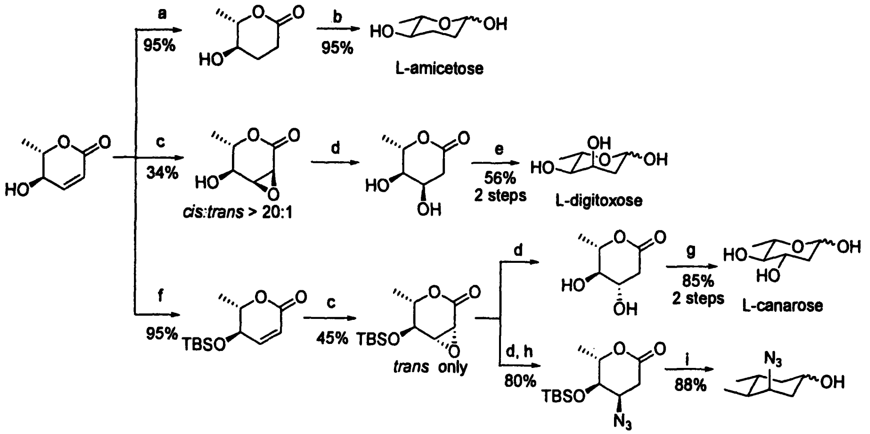
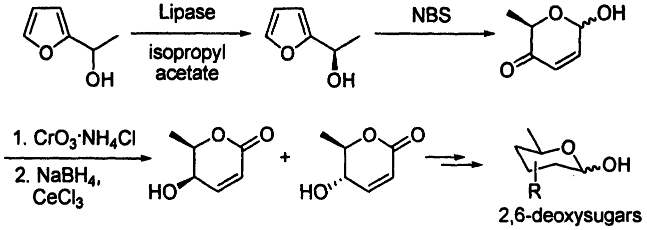

a
95%
b
95%
L-amicetose
c
34%
cis:trans > 20:1
d
e
56%
2 steps
L-digitoxose
d
g
85%
2 steps
L-canarose
f
95%
c
45%
trans only
d, h
80%
i
88%

Scheme 3. a) H_2, Pd/C; b) DIBAL-H; c) NaClO, Pyridine; d) $NaBH_4$, PhSePh, AcOH; e) $NaBH_3CN$; f) TBSCl, imidazole; g) TBAF then $NaBH_3CN$; h) DPPA, PPh_3, DEAD; i) TBAF then DIBAL-H.


Lipase
isopropyl acetate
NBS
1. CrO3·NH4Cl
2. NaBH4, CeCl3
2,6-deoxysugars

Scheme 4. Alternative enzymatic synthetic pathway of 2,6-dideoxysugar.

Recently, we developed another synthetic pathway by enzymatic resolution of 1-(2-furyl)ethanol followed by Achmatowicz rearrangement (Scheme 4) *(14)*. In this pathway, furanaldehyde, an agrobased product, is used as starting material for preparation of the chiral α,β-unsaturated lactones. As an alternative way to construct the desired α,β-unsaturated lactones, this strategy is more economic and more practical for large-scale manipulations than that one shown in scheme 2. So far, 20 uncommon sugars were obtained in large quantity (Figure 1) by using these two strategies in our group.

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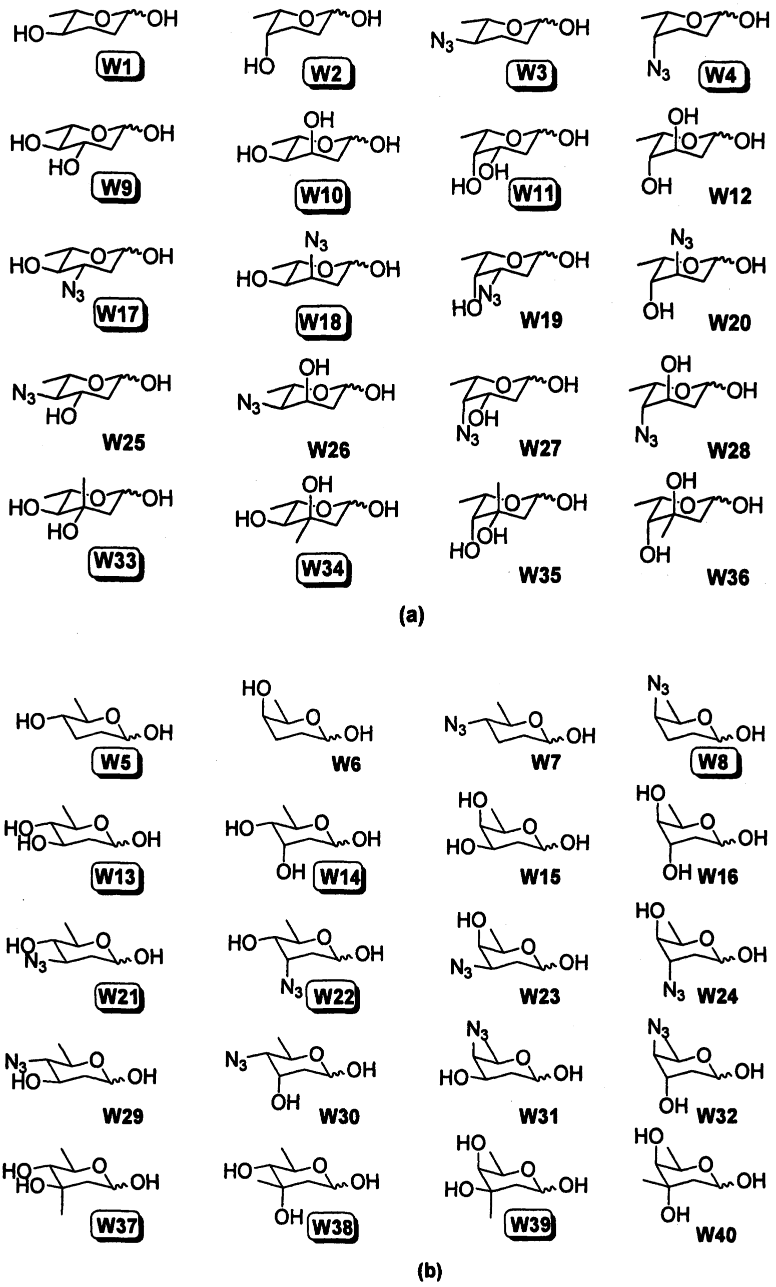
W1
W2
W3
W4
W9
W10
W11
W12
W17
W18
W19
W20
W25
W26
W27
W28
W33
W34
W35
W36
(a)
W5
W6
W7
W8
W13
W14
W15
W16
W21
W22
W23
W24
W29
W30
W31
W32
W37
W38
W39
W40
(b)

Figure 1. Uncommon sugar library: (a) L-Sugars; (b) D-Sugars (Boxed compounds are currently available in Wang's lab).

Uncommon Sugar Conjugates to Natural Products

Carbohydrate moieties are frequently found in the structure of biologically active natural products. The function of the sugar moiety can be multifold ranging from affecting the pharmacokinetics of the drug to directly interacting with cellular targets of proteins, RNA and DNA. Our preliminary research indicated that aglycon of rebeccamycin and daunorubicin without sugar moieties also lost their anticancer activities. Many of the sugar moieties in natural products are "uncommon" sugars. It has been repeatedly validated that altering the crucial sugar structure of natural products can enhance their pharmacological activity *(3-5,15-23)*. The approach of exchange or substitution of the carbohydrate moiety in natural products is call "glycorandomization" *(24,25)*.

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Rebeccamycin analogs with uncommon sugars

Rebeccamycin (**Reb**) is an antitumor natural product isolated from Saccharotrix aerocolonigenes ATCC 39243 *(26,27)*. All reported rebeccamycin analogs with potent antitumor activity share a common indolocarbazole core coupled with a single β-glycoside moiety *(28-30)*. Sugar moieties in rebeccamycin analogs are considered as DNA-minor groove binding units (Figure 2), which ensure high affinity with DNA, and topo I poisoning ability of rebeccamycin *(31-33)*.

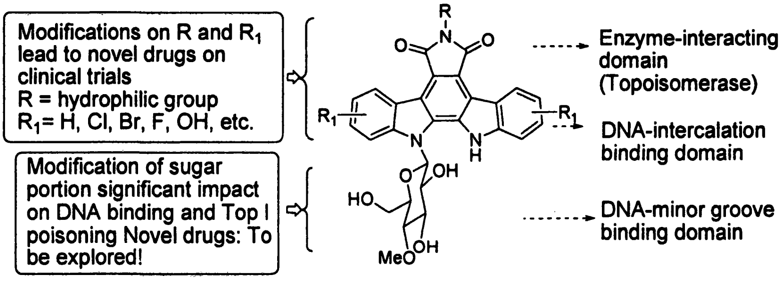
Modifications on R and R_1 lead to novel drugs on clinical trials
R = hydrophilic group
R_1= H, Cl, Br, F, OH, etc.
Modification of sugar portion significant impact on DNA binding and Top I poisoning Novel drugs: To be explored!
R
O
N
O
R_1
R_1
N
N
H
O
OH
HO
MeO
OH
Enzyme-interacting domain (Topoisomerase)
DNA-intercalation binding domain
DNA-minor groove binding domain

Figure 2. SAR of rebeccamycin.

Many studies suggest that altering glycosylation patterns on indolocarbazole has a high potential for generating novel chemotherapeutics *(34-36)*. To explore the importance of OH group at the 2,3,6 positions in the sugar and the impact of an uncommon aminosugar, three uncommon sugars 2-deoxy-L-glucose, 2,6-dideoxy-L-glucose and 3-amino-2,6-dideoxy-L-fucose were selected as glycosyl donors. As shown in Figure 3, a series of rebeccamycin analogs with various

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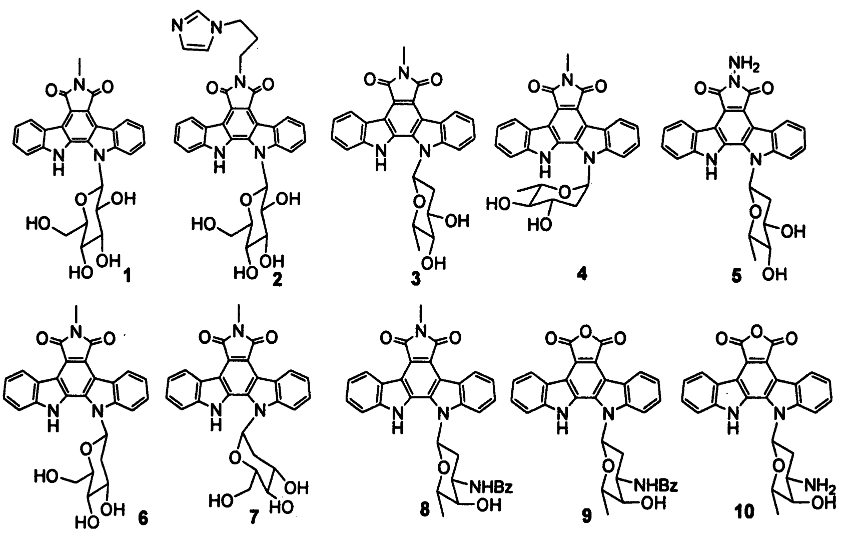


Figure 3. Synthesized rebeccamycin analogs.

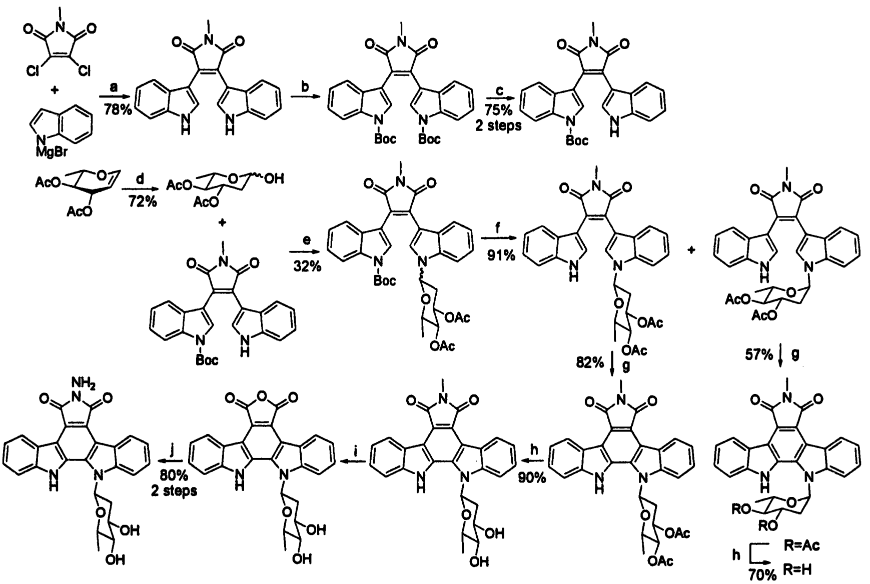
uncommon sugars and substitution on the imide nitrogen were prepared using the strategy shown in scheme 5 *(37)*.

The cytotoxicities and topoisomerase I inhibition of these compounds (Figure 3) were tested in cancer cells in comparison with their parent compounds. As shown in Table I, those compounds with glucose moiety (**1**, **2** and **Reb**) are more active than compounds with 2-deoxyglucose (**6** and **7**) or with 2,6-dideoxyglucose (**3** and **4**) against colon cancer cells (SW620) and leukemia cells (K562). Compounds with 2,3,6-trideoxyglucose (**8**) are the least active. These data indicate that 2-OH, 3-OH and 6-OH groups in the sugar moieties, rather than the modifications in the imide structure, play crucial roles in maintaining their anticancer activity. The better activities of compounds **1**, **2** and **6** indicate that the 6-OH may be more important than other hydroxyl groups in the sugar moiety. The 6-OH group on the carbohydrate residue may form a hydrogen bond with the indole NH group, which would maintain the

Table I. Anticancer Activity (IC50) in Two Cancer Cell Lines (μM).

	Aglycon	1	2	3	4	5	6	7	8	9	10	Reb
SW620	>100	5	2	24	34	>100	13	32	>100	76	>100	4
K562	40	3	4	26	19	>100	12	12	34	53	55	6

Source: Reproduced with permission from reference 37. Copyright 2005.)



Scheme 5. Reagents and conditions: a) Et_2O/toluene, 88 °C, 24 h; b) Boc_2O, DMAP, THF, rt, 1 h; c) TBAF, THF, reflux, 8 h; d) $LiBr \cdot H_2O$, AG 50W-X2 resin, CH_3CN, rt, 15 min; e) Ph_3P, DEAD, THF, -78 °C, 15 h ($\alpha:\beta = 1:2.5$); f) 88% HCO_2H, rt, 6 h; g) hν, *air, I_2, benzene, 8 h; h) TBAF, THF, reflux, 15 h; i) EtOH/toluene (1:3), 48% KOH, rt, overnight; then 10% citric acid; j) $N_2H_4 \cdot H_2O$.*

carbohydrate in a fixed conformation for optimal activity and interaction with the topoisomerase I-DNA complex.

As shown in Figure 4, compound **1** with glucose moiety showed similar strong topoisomerase inhibition as that of CPT at the same molar concentration, However, compound **6** with 2-deoxy-D-glucose moiety gave a very weak topo I inhibition while compound **3** with 2,6-dideoxy glucose moiety was inactive. This indicates that the 2-OH and 6-OH of the sugar moiety also modulate the topoisomerase I activity in cancer cells, which is worthy of further investigation with various modifications in the sugar moiety. Therefore, the modifications of rebeccamycin with uncommon sugars may provide a new class of anticancer compounds.

Daunorubicin analogs with uncommon sugars

The anthracycline quinine antibiotics daunorubicin (**DNR**) and doxorubicin (**DOX**) (Figure 5) are potent antitumor agents against a variety of human solid

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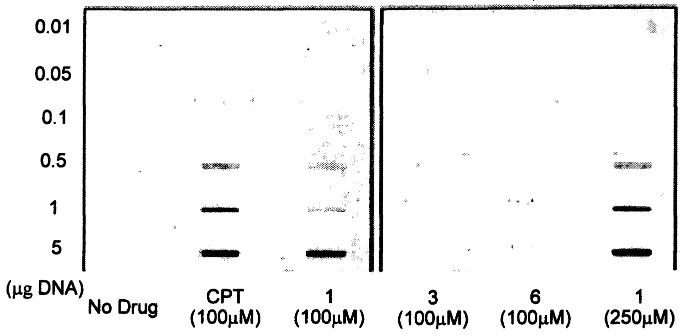
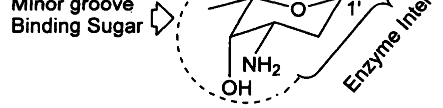


*Figure 4. Standard ICT assay in HeLa cells comparing camptothecin (CPT) with Rebeccamycin-sugar derivatives **1**, **3**, and **6**. Exponentially growing HeLa cells were incubated with the compounds indicated to the right of the blot for 30 min at 37 °C, followed by sarkosyl lysis and a standard ICT assay (CsCl gradient separation of DNA, recovery of DNA and spotting from 0.01 to 5 ìg of DNA on the blot). The blot was probed with anti-topo I antibody. Signals were developed using ECL and a short (1 min) exposure. (Reproduced with permission from reference 37. Copyright 2005.)*


DNA intercalating Domain
Minor groove Binding Sugar
Enzyme Interacting Domain
O
OH
OH
O
OMe
O
OH
O
O
NH2
OH
R
1
4
6
7
10
12
13
5'
1'
D
C
B
A

R = CH_3 Daunorubicin
R = CH_2OH Doxorubicin

*Figure 5. SAR of **DNR** and **DOX**.*

tumors and leukemia *(38)*. In addition to DNA intercalation, topoisomerase II (topo II) is considered as the primary cellular target of anthracyclines *(39,40)*. The well-accepted mechanism of action is through interaction with DNA-topo II by stabilization of topo II-DNA-drug ternary complex, which also triggers apoptosis. It is noted that DNA binding and interaction are necessary, but not sufficient, for topo II poisoning *(41)*. In fact, the external (non-intercalating) sugar moieties and the cyclohexane ring A are recognized as crucial moieties for therapeutic efficacy of anthracyclines. The sugar moiety of anthracyclines serves as a minor groove binder of DNA *(42,43)*. Indeed, modifications on the sugar structures have led to the second generation of doxorubicin analogs as monosaccharides such as epirubicin, valrubicin and pirarubicin.

The third generation anthracycline analogs with disaccharide, such as MEN 10755 (Figure 6) *(44-46)*, possess a 2-deoxy fucose linked to the aglycon and daunosamine as the second sugar unit. Extensive studies for the SAR of MEN 10755 reveal that the axial orientation of C-O-1 bond of the second sugar daunosamine is critical for the topo II poisoning ability. Indeed, glycoside with equatorial configuration is ineffective in topo II poisoning *(47,48)*. The presence of 4-methoxy group in the second sugar unit dramatically decreases its cytotoxicity compared to anthracycline monoglycosides due to the reduced topo II poisoning *(47,48)*. It is conceivable that the axial orientation is optimal for the interactions of MEN 10755 with the DNA-topo II complex only in the absence of methoxy group, and this is confirmed by the crystal structure of the complex between MEN 10755 and hexanucleotide (CGATCG) *(49)*. This structure is similar to previously crystallized anthracycline-DNA complexes, while MEN 10755 showed two different DNA binding sites. In one binding site, the disaccharide resides in the DNA minor groove; in the other binding site, the

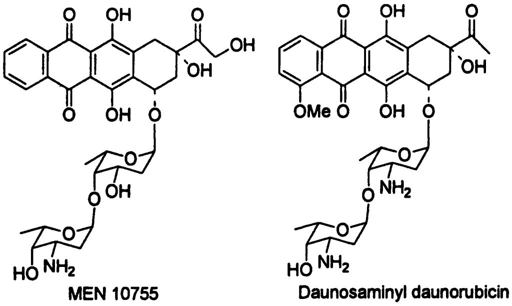


Figure 6. Disaccharide anthracycline analogs.

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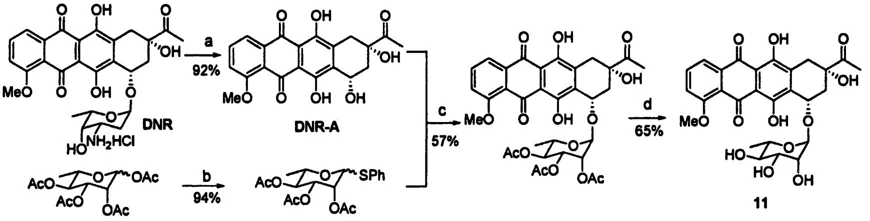
second sugar protrudes from the DNA helix and is linked to guanine of another DNA through hydrogen bonds. This peculiar behavior suggests that the second sugar may interact with other cellular target such as topoisomerase. MEN-disaccharides is capable of stimulating topo-I mediated DNA breakages *(50)*.

Monosaccharide daunorubicin analogs

Previous research has indicated that the linkage between the sugar moiety and the aglycon is very important. Only the α-linked monosaccharide daunorubicin analogs are biologically active. Therefore, the uncommon sugars were introduced stereoselectively. Thioglycosides were used as glycosyl donors because of their high stability *(51-53)*. The promoter system $AgPF_6$/TTBP is efficient for the α-selective glycosylation *(54)*. Taking advantage of our uncommon sugar library, six desired α-linked monosaccharide daunorubicin analogs were synthesized using the method as shown in Scheme 6 *(55)*.



Scheme 6. Reagents and conditions: a) 0.2 M HCl, 90 °C, 1 h; b) PhSH, $BF_3{\cdot}Et_2O/CH_2Cl_2$, 0 °C, 4 h; c) TTBP, $AgPF_6/CH_2Cl_2$, 4 Å MS, 0 °C, 4 h; d) 0.1 M NaOH / THF, 0 °C, 6 h.

The cytotoxicities of these six compounds (Figure 7) were examined in colon cancer cell line SW620 cells. The results in Table II indicated that the aglycon, **DNR-A**, exhibited 70 to 100-fold lower cytotoxicity than daunorubicin derivatives with various uncommon sugars. This suggests that sugar structure in daunorubicin plays a critical role in determining its anticancer activity.

Compound **14** with 3'-OMe terminal 2,6-dideoxysugar showed very potent cytotoxicity with IC_{50} of 0.1 μM. Importantly, compared to compounds **12** and **13** (with *axial*-3'-OMe or *axial*-3'-OH group), **14** (with an *equatorial*-3'-OMe group) showed 10 to 20-fold higher anticancer activity. This suggested that the *axial*-3'-substituent in sugar (such as in compounds **12** and **13**) may interfere daunorubicin binding to DNA.

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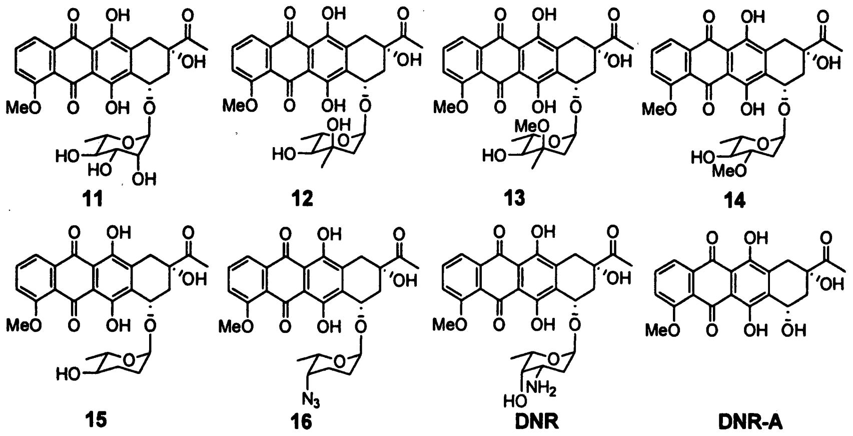

Figure 7. Synthetic monosaccharide daunorubicin analogs.

Table II. Cytotoxicity (IC_{50}) of compounds 11-16 against colon cancer SW620 cells.

Compounds	11	12	13	14	15	16	DNR-A	DNR
IC_{50} in SW620 (μM)	0.26	>1	>1	0.1	0.35	>1	>2	0.033

Compound **14** with 3'-OMe terminal 2,6-dideoxysugar showed very potent cytotoxicity with IC_{50} of 0.1 μM. Importantly, compared to compounds **12** and **13** (with *axial*-3'-OMe or *axial*-3'-OH group), **14** (with an *equatorial*-3'-OMe group) showed 10 to 20-fold higher anticancer activity. This suggested that the *axial*-3'-substituent in sugar (such as in compounds **12** and **13**) may interfere daunorubicin binding to DNA.

Compounds **11** and **15** with *equatorial*-4'-OH showed similar activity, while compound **16** (substituted 4'-OH with *axial*-4'-N_3 in the sugar moiety) lost its cytotoxicity. This suggests that 4'-OH in the sugar may also be important for sugar containing anthracycline as an anticancer agent. Further SAR studies are required to clarify this finding.

From the published results *(41)*, it is found that the α-face of the sugar is lying towards the DNA base pairs when binding to DNA. Therefore, increasing the hindrance of the α-face of the sugars may lead to the decreased cytotoxicity against cancer cells. All these results suggest that the 2,6-dideoxy sugars are the

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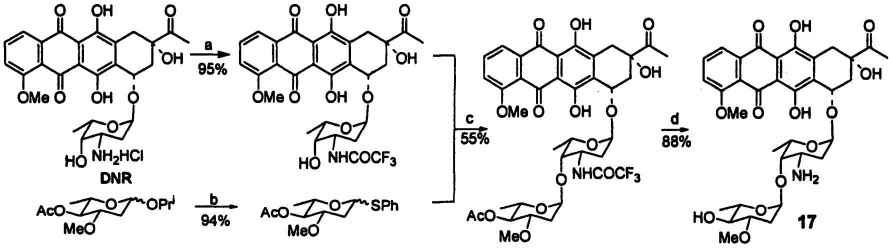
best choice for making biologically active daunorubicin analogs than 6-deoxysugars, 2,3,6-trideoxysugars, or 2,3,4,6-tetradeoxysugars. It also indicates that the sugar moiety may not be just simply lying in the minor groove of the DNA, the functional group of the sugar would rather interact with the base pairs for the biological activity of daunorubicin analogs.

Disaccharide daunorubicin analogs

Natural anthracycline analogs isolated from *Streptomyces sp.* often contain two or more sugars connected by axial (1→4) linkage. The anthracycline disaccharides or oligosaccharides invariably possess amino sugar as the first moiety attached to the aglycon *(38,56)*. For instance, daunosaminyl daunorubicin (Figure 6), which is isolated from fermentation, contains two daunosamines connected through α (1→4) linkage. The structure of the terminal sugar and sugar chain length have been shown to significantly influenced DNA binding, topoisomerase poisoning, and anticancer efficacy *(57)*.

In the present study, we designed novel disaccharide daunorubicins with uncommon sugars as terminal sugar moieties to reveal their function in anticancer activity. The optimal configuration of the disaccharide in daunorubicins analogs should be an uncommon sugar linked to the 4-position of the daunosamine through α (1→4) linkage. Herein, eight desired disaccharide analogs were synthesized through the same protocol used for the monosaccharide analogs (Scheme 7) *(58)*.



Scheme 7. Reagents and conditions: a) $(CF_3CO)_2O$ / pyridine, -20 °C, 15 min; b) PhSH, $BF_3 \cdot Et_2O$ / CH_2Cl_2, 0 °C, 4 h; c) TTBP, $AgPF_6$ / CH_2Cl_2, 0 °C, 2 h; d) 0.1 M NaOH / THF, 0 °C, 6 h.

The cytotoxicities of these compounds (Figure 8) were examined in leukemia cell line K562 cells and colon cancer cell line SW620 cells with MTS

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assay. The ability of these compounds to mediate topo II cleavage complexes in vivo was also tested using ICT bioassay in Hela cells.

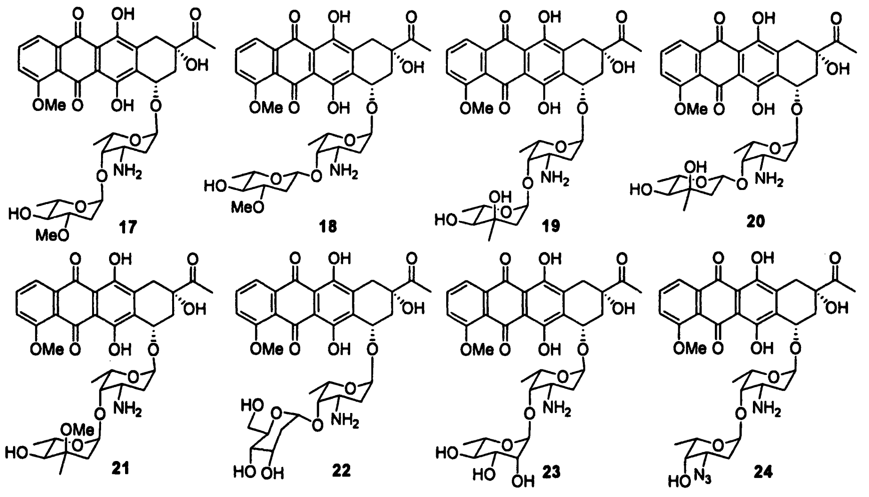

Figure 8. Synthetic disaccharide daunorubicin analogs.

The biological activities of these compounds in two cancer cell lines (Table III) show that compounds **17**, **19**, **21** and **24** with various terminal 2,6-dideoxysugars showed 30-60 fold better cytotoxicity compared to compounds **22** and **23** which contain either 2- or 6-deoxy sugar, while structural modification on other sites in the second sugar moiety (such as 3-position, –OH or –OMe) did not affect the activity significantly. This suggests that 2,6-dideoxysugars may be required for anticancer activity of daunorubicin and its derivatives. In addition, the results show compound bearing α-configuration of the terminal 2,6-dideoxy-sugar moiety is 35-50 fold more potent than compounds with β-configuration. Figure 9 showed the anticancer activity of these compounds correlated with their ability to target topo II mediated DNA damage in vivo. It indicated that daunorubicin analogs with α-linked terminal 2,6-dideoxy sugars (compounds **17** and **19**) showed higher topo II targetabililties than their β-linked counterparts (compounds **18** and **20**), while compounds with 2-deoxy (compound **22**) or 6-deoxy (compound **23**) terminal sugar did not show any topo II inhibition. These results reveal that sugar moieties and conformation may be important for their anticancer activity and topo II targeting. The second sugar 2,6-dideoxy with alpha-linkage to the first sugar of daunorubicin disaccharide is preferred to exhibit anticancer activity.

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Table III. Anticancer Activity (IC_{50}) of Synthesized Compounds in Two Cancer Cell Lines.

Compounds	17	18	19	20	21	22	23	24	DNR
IC_{50} in K562 (nM)	39.5	45.8	44.3	1531.6	21.0	1378.3	>4000	31.3	15.6
IC_{50} in SW620 (nM)	18.2	38.9	21.7	>1000	11.4	>1000	>1000	60.3	8.6

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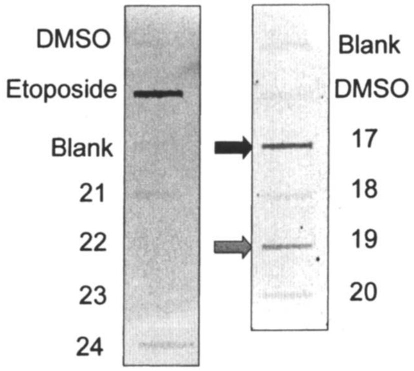

Figure 9. Inhibition of topoisomerase II by compound 17-24. Exponentially growing Hela cells was incubated with 50uM etoposide or tested compounds for 30 minutes at 37°C. DNA and denatured proteins were fractionated by CsCl gradient centrifugation. The bottom fraction was collected. Each fraction (50 μl) was slotted onto a Hybond ECL membrane and the blot probed with anti-topo II antibody. The immunoblotsignals were visualized by chemiluminescence. (Reproduced with permission from reference 58. Copyright 2005.)

Conclusion and Outlook

A wealth of data have shown that sugar moiety in these compounds play a critical role for their anticancer activity. Although a glycorandomization approach may initially seem empirical (not totally structurally based drug design), structure-activity-relationship (SAR) from the screening of the synthetic drug analogs will provide valuable guidelines for further drug development. For active candidates, further studies for interaction of the drug

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with their cellular targets (DNA, topoisomerases I & II,) will reveal new insights into the mechanism of actions for these classes of anticancer drugs.

One of the challenges in cancer chemotherapy is the development of multiple drug resistances (MDR), which limits the clinical application of many anticancer compounds such as rebeccamycin and daunorubicin. Thus, development of rebeccamycin and anthracycline analogs that can overcome MDR would have great biomedical implications. By screening a series of rebeccamycin and daunorubicin derivatives with different uncommon sugar from our library, we will continue to study these compounds for their anticancer activity against drug-resistant cancers.

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Chapter 3

Plant Cell Wall Glycans: Chemical Synthesis of the Branched Sugar Aceric Acid

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In connection with studies on the synthesis of complex cell wall glycans, we have developed effective syntheses of the novel branched sugar aceric acid and its C-2 epimer. Control of asymmetry in the installation of the key tertiary centers was effected by either asymmetric dihydroxylation of an appropriate alkene derivative or by thiazole addition to the corresponding ketone.

Background

Whilst much attention has been paid to medically related issues (*1*) our appreciation of the molecular detail of plant glycobiology is still rather rudimentary. This is rather surprising given the undoubted capacity of plants to perform carbohydrate chemistry. Completion of sequencing of the Arabidopsis genome in 2001 led to the observation that perhaps as much as ~5% of the entire genome may code for some aspect of carbohydrate chemistry (*2*). "Why are there so many carbohydrate-active enzyme-related genes in plants?"(*3*) At least partly because all higher plant cells are surrounded by polysaccharide-rich primary cell walls, which are believed to play a crucial role in regulating plant structure, growth and resistance to disease (*4*). It is not yet well understood how different

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components of the cell wall are organized, how they interact with each other and what the implications are of these interactions in relation to a wide range of cell wall functions (*5*). Neither has it proved straightforward to identify potential plant cell wall glycosyltransferase-encoding genes, although improvements in informatics look promising (*6,7*). A comprehensive description of the primary wall will require much research at the molecular level; availability of well-characterized saccharide fragments in quantity would greatly aid further progress. Towards this end, we have begun a program of chemical synthesis of plant cell wall glycans, with an initial focus on one of the most complex oligosaccharides found in Nature, namely rhamnogalacturonan-II (RG-II; Figures 1 and 2).

RG-II, a so-called "mega-oligosaccharide"(*8*) that was first reported in 1978 (*9*), is conserved in primary cell walls of all higher plants. Structural studies have revealed its main features (*10-12*): it has a main backbone of 7 to 9 1,4-linked α-D-Gal*p*A residues and four structurally different oligoglycosyl side chains incorporating in total 11 different kinds of sugar residue linked by 20 different types of glycosidic linkage (Figure 1). Depending on the source of RG-II, structural variations associated with the lack of some terminal monosaccharide residues are evident (*13*), but the overall architecture of RG-II is essentially conserved throughout higher plants. It is not at all clear why evolutionary pressure has not resulted in simplification of RG-II structure. It remains to be seen if specific structural features of RG-II render it 'fit for purpose', or whether its structure has simply been retained because of its resistance to biological degradation (despite the fermentation process, RG-II is found at high levels in red wine, for instance) (*14*). It is conceivable that the highly branched nature of the RG-II structure is important in this regard (*15,16*). In addition, RG-II has the unique potential to couple polysaccharide chains in the plant cell wall by virtue of its ability to form dimers that are cross-linked by a borate diester (*17,18*) (Figure 2).

It is believed that the apiofuranose (Api*f*) residue of side chain A, but not that of side-chain B, is involved in RG-II dimerization. Recently mutant Arabidopsis *mur1* strains, which are defective in L-fucose biosynthesis, have been shown to result in small changes in chain A of RG-II: the L-fucose residue is replaced by L-galactose. This seemingly minor modification in a complex oligosaccharide structure results in a significant reduction in RG-II dimer formation and has a dramatic impact of plant morphology(*19*). It is not yet clear which structural features of RG-II are key to borate ester formation and stability.

Recent work

In order to investigate RG-II-related borate complexation phenomena, recent studies by others and us have detailed synthesis of fragments **1-3** (*20*), **4** (*21*), **5** (*22*) and **6** (*23*) of RG-II side chain A (Figure 3).

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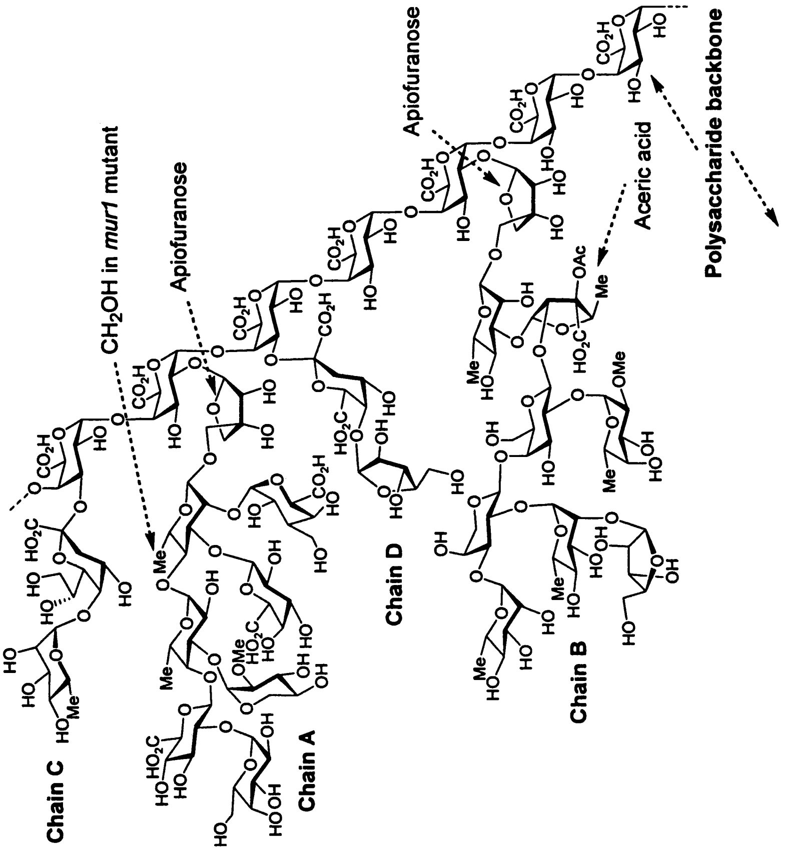
CH$_2$OH in *mur1* mutant
Apiofuranose
Apiofuranose
Aceric acid
Polysaccharide backbone
Chain A
Chain B
Chain C
Chain D

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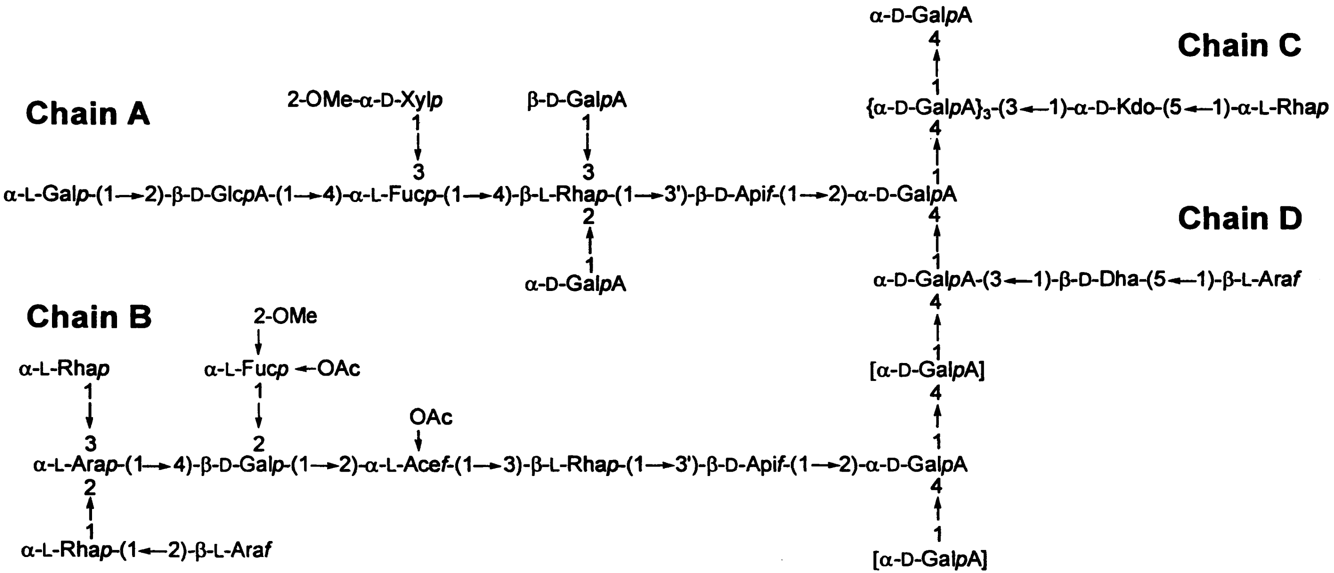
Chain A
2-OMe-α-D-Xylp
β-D-GalpA
α-L-Galp-(1→2)-β-D-GlcpA-(1→4)-α-L-Fucp-(1→4)-β-L-Rhap-(1→3')-β-D-Apif-(1→2)-α-D-GalpA
α-D-GalpA
Chain B
2-OMe
α-L-Rhap
α-L-Fucp ←OAc
OAc
α-L-Arap-(1→4)-β-D-Galp-(1→2)-α-L-Acef-(1→3)-β-L-Rhap-(1→3')-β-D-Apif-(1→2)-α-D-GalpA
α-L-Rhap-(1←2)-β-L-Araf
α-D-GalpA
Chain C
{α-D-GalpA}$_3$-(3←1)-α-D-Kdo-(5←1)-α-L-Rhap
Chain D
α-D-GalpA-(3←1)-β-D-Dha-(5←1)-β-L-Araf
[α-D-GalpA]
[α-D-GalpA]
1
2
3
4

Figure 1. Structure of rhamnogalacturonan-II and abbreviated representation of its sequence, highlighting side chain structures and key branched sugar residues

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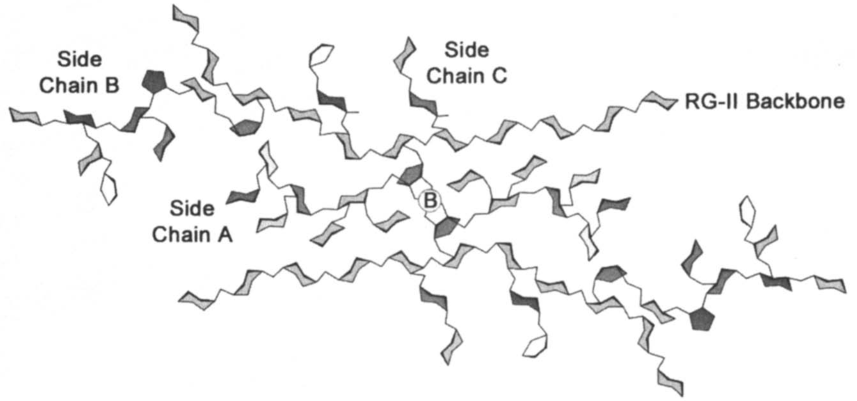
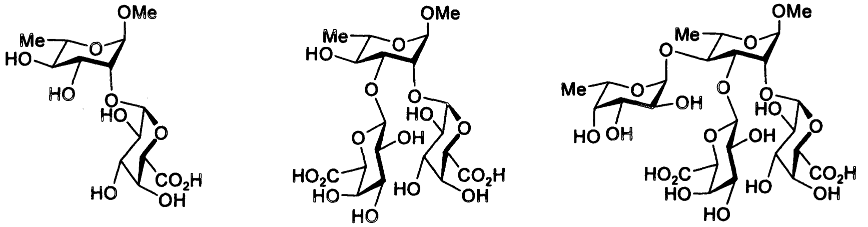


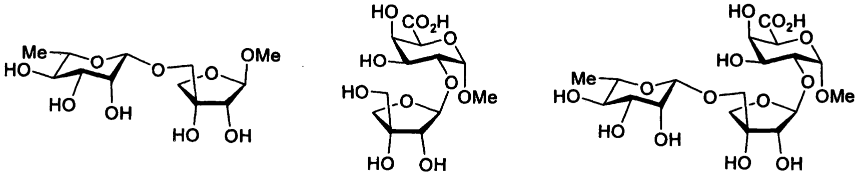
Figure 2. Cartoon representation of RG-II dimer.



1 GalpA(α1-2)Rhapα-OMe

2 GalpA(α1-2)[GalpA(β1-3)]Rhapα-OMe

3 GalpA(α1-2)[GalpA(β1-3)]-[Fucp(α1-4)]Rhapα-OMe



4 Rhap(β1-3')Apifβ-OMe

5 Apif(β1-2)GalpAα-OMe

6 Rhap(β1-3')Apif(β1-2)GalpAα-OMe

Figure 3. RG-II side chain A fragments synthesized to date (21-24).

In addition, we have begun syntheses of RG-II side chain B fragments in order to determine why, even though it possesses the core Rha*p*β(1-3')Api*f*β(1-2)-Gal*p*A trisaccharide in common with side chain A (Figure 2), side chain B does not apparently complex borate. Reading from the poly-Gal*p*A backbone, the first point of difference between side chains A and B lies in the substitution of the β-linked rhamnose residue (Figure 2). In side chain B, the rhamnose residue is substituted by a unique branched sugar, aceric acid (Ace*f*) which has neen found nowhere else in Nature. In order to conduct studies on chain B, a synthesis of aceric acid and its derivatives was needed (Figure 4, top line).

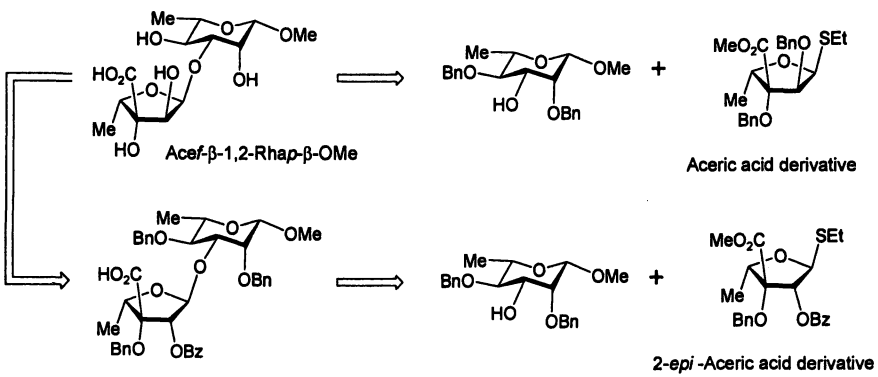


Figure 4. Disconnections of the Acef-β-1,2- Rhap component of side chain B

Taking into account the need to incorporate a 1,2-*cis*-β-linked aceric acid unit into side chain B structures (Figure 4), we also chose to prepare 2-*epi*-Ace*f* and derivatives (Figure 4, bottom line). This epimer would allow the use of a participating group on C-2 to bring about 1,2-*trans*-glycosylation, followed by inversion of the C-2 stereocenter to access the 1,2-*cis*-linked Ace*f* residue. No previous synthesis of Ace*f* or its C-2 epimer had been reported prior to our work (24).

Synthetic approaches

Introduction of C-3 branch point functionalities that can be later deblocked and/or oxidized to form a carboxylic group is possible via at least two different routes (Figure 5). One commonly used approach in the synthesis of branched-chain sugars involves addition of a nucleophile to an ulose (*25*). However, use of 2-dithianyllithium (*26*), vinyl magnesium bromide (*27*) or 2-furyl lithium (*28*) is not likely to lead to the correct C-3 stereochemistry since the preferred direction of their approach to ulose 7 will be from the sterically less demanding *exo*-face

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of the trioxabicyclo[3.3.0]octane-based ketone. On the other hand, asymmetric dihydroxylation (*29*) of alkene **8**, which can be prepared from 3-ulose **7** via a Wittig reaction, presents an alternative route to aceric acid. In this approach, dihydroxylation should proceed from the more accessible *exo*-face of the bicyclic alkene **8**, affording a branched-chain derivative with the correct orientation of the 3-*C*-hydroxymethyl group relative to the furanose ring. Selective oxidation of the primary OH group may then provide the required 3-*C*-carboxy-derivatives. Due to its simplicity, this latter approach was investigated first.

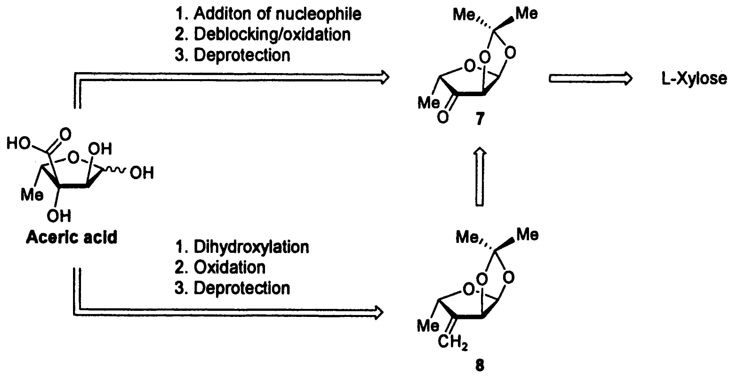

Figure 5. Retrosynthetic synthesis of aceric acid.

Dihydroxylation route to aceric acid

6-Deoxy-3-ulofuranose **7** was synthesized in five steps, as shown in Figure 6. This unstable ketone was then converted into the 3-*C*-methylene derivative **8** in 70% yield by reaction with $H_2C{=}PPh_3$, followed by dihydroxylation of this compound using Sharpless' conditions (*29*) to afford the same single stereoisomer **9**, regardless of whether AD mix α or AD mix β was used.

One can anticipate that the preferred face for dihydroxylation of olefin **8** is *exo* with respect to the bicyclic ring system, and therefore the stereochemistry of the reaction product **9** was tentatively assigned as L-*xylo*. However, initial 2D NOESY NMR spectroscopy data for **9** were inconclusive when analyzed in the absence of data for stereoisomeric compounds. This left the assignment of C-3 stereochemistry ambiguous for compound **9**. Furthermore, literature precedent (*30*) shows that dihydroxylation of alkene **10** results in diol **11**, having the newly formed hydroxymethyl group *anti* with respect to the isopropylidene group, indicative of dihydroxylation of the more hindered *endo* face of the alkene (Figure 7).

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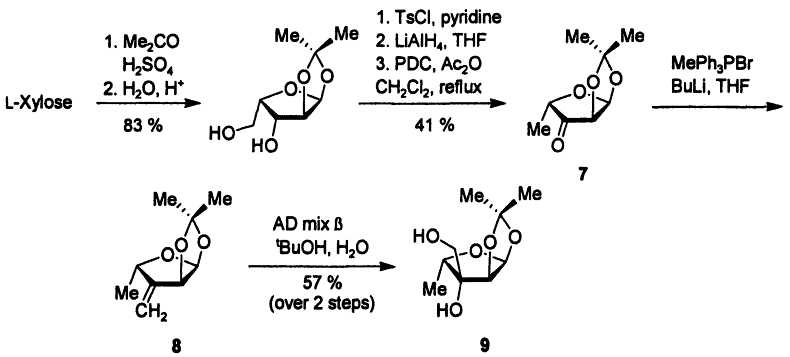
L-Xylose
1. Me2CO
H2SO4
2. H2O, H+
83 %
1. TsCl, pyridine
2. LiAlH4, THF
3. PDC, Ac2O
CH2Cl2, reflux
41 %
7
MePh3PBr
BuLi, THF
AD mix ß
tBuOH, H2O
57 %
(over 2 steps)
8
9

Figure 6. Synthesis of branched sugar derivative 9 via a dihydroxylation approach.

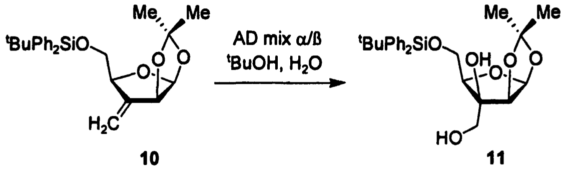
AD mix α/ß
tBuOH, H2O
10
11

Figure 7. Dihydroxylation of 3-C-methylene furanose derivative 10 (30).

Due to the potential ambiguity in the structure of branched sugar **9** an alternative approach to installing the requisite chiral quaternary centre was considered.

Thiazole addition approach to aceric acid

An example of highly stereoselective *endo*-addition to a bicyclic 3-ketofuranose was reported (*31*) in the reaction of diacetoneglucose-derived ketone **12** with 2-(trimethylsilyl)thiazole (2-TST), leading to the branched-chain derivative **13** (Figure 8).

It was proposed that this reaction relies on thermodynamic control of the addition of an *in situ*-generated chiral thiazolium anion, incorporating ketone **12**, to another molecule of **12**. It is not clear what role, if any, the bulky 5,6-*O*-isopropylidene group, which sits on the *exo*-face of the bicycle, plays in directing *endo*-addition to ketone **12**. However, the relative orientation of this bulky

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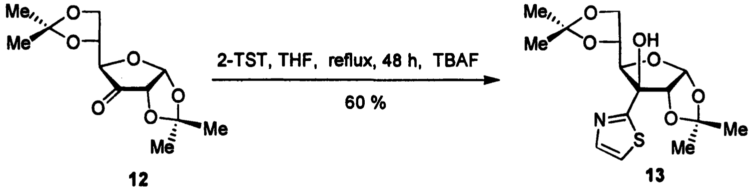

Figure 8. Stereoselective addition of 2-TST to 3-ketofuranose 4 (31).

substituent mirrors that of the *exo*-disposed C-5 methyl group in ketone **7**, so encouraging us to explore the reaction of ketone **7** with 2-TST.

To investigate a thiazole addition approach to aceric acid, 3-ulose **7** was treated with 2-TST in THF, under thermodynamic control, followed by desilylation with TBAF. This reaction sequence led to a 5:1 mixture of *endo*- and *exo*- adducts **14** and **15**, respectively, in an overall yield of 70% (Figure 9, *Method A*). In contrast, 2-thiazolyl lithium addition to compound **7** gave only the adduct **15** arising from sterically controlled *exo*-addition to the trioxabicylo[3.3.0]octane ring system in an unoptimized 40% yield (Figure 9, *Method B*). Both isomeric adducts **14** and **15** were benzylated to afford 3-*O*-benzyl derivatives **16** and **17**, respectively. The stereochemistry of thiazole addition to 3-ulose **7** was established by X-ray structural analysis of compounds **14** and **17** (Figure 10), which revealed that **14** has the required C-3 configuration for aceric acid synthesis.

A plausible mechanism to account for the stereochemical outcome of the 2-TST-mediated addition process is outlined in Figure 11.

This process is characterized by thermodynamic equilibrium between zwitterion intermediates A and B in which an intermediate A preceding *endo*-

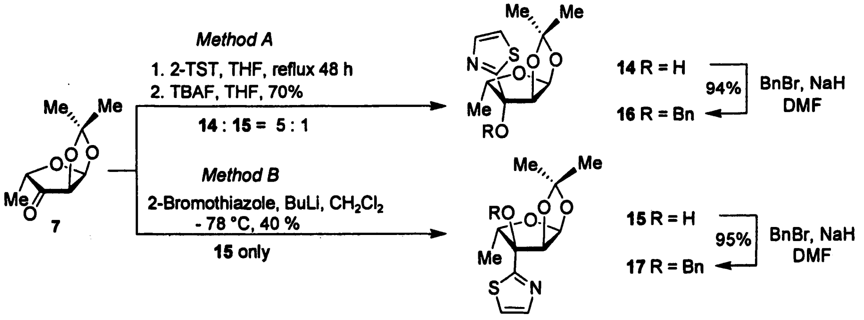

Figure 9. Thiazole-based addition to bicyclic ketone 7 to install a key tertiary stereocentre.

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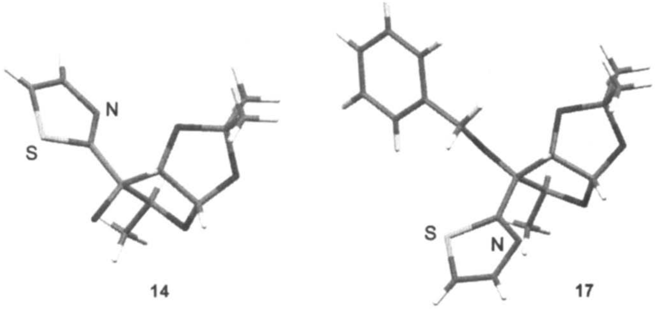

*Figure 10. X-ray crystal structures of endo-thiazolyl derivative **14** and 3-O-benzylated exo-thiazolyl derivative **17**.*

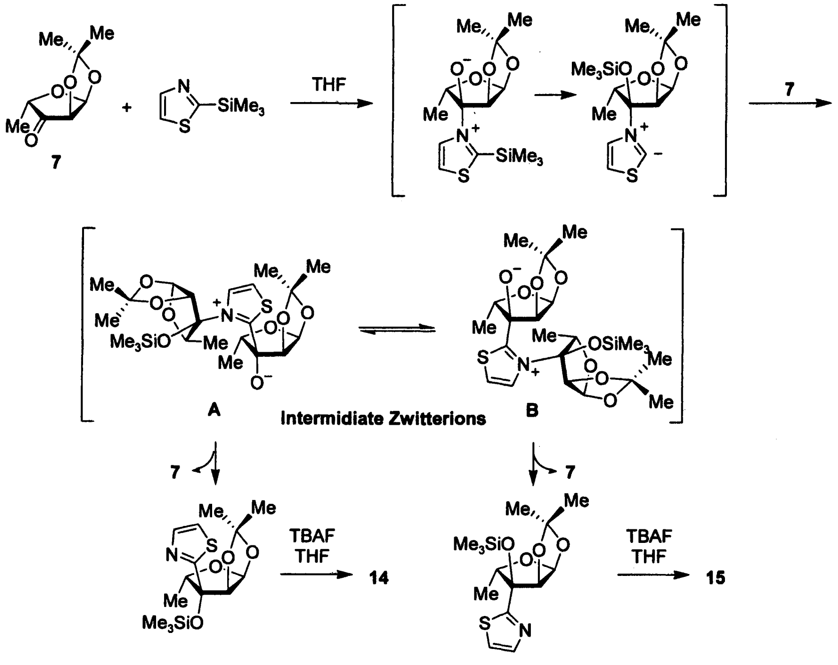
7
THF
7
A
Intermidiate Zwitterions
B
7
7
TBAF
THF
14
TBAF
THF
15

Figure 11. Proposed mechanism for 2-TST addition to 3-ketofuranose 2 [adapted from (31)].

addition product **14** prevails. The reason for the higher stability of this intermediate may be due to a more favourable orientation of C-C bond linking furanose and thiazole rings in **A** compared to **B**. Indeed, examination of the X-ray crystal structures of *exo*-thiazolyl derivative **14** and *endo*-thiazolyl derivative **17** revealed that in both of these compounds the furanose rings have ^{4}E conformations leading to pseudo-equatorial orientation of the thiazolyl substituent in **14** and its pseudo-axial orientation in **17**. It seems reasonable to suggest that the same trend exists in the corresponding intermediates **A** and **B**, making intermediate **A** with a sterically less demanding bulky equatorial group more stable than intermediate **B**.

Thiazole adduct **16** was subsequently converted to aldehyde **18** using a 3-step, one-pot reaction (*32*), followed by oxidation to acid **19** and deprotection to give aceric acid **21** (Figure 12). Synthetic compound **21** prepared in this manner gave spectroscopic data identical to those for authentic aceric acid (*33*). To tie in with the dihydroxylation chemistry described earlier, aldehyde intermediate **18**, produced by the 2-TST addition route, was converted to alcohol **9** by NaBH$_4$ reduction followed by removal of 3-*O*-benzyl group by catalytic hydrogenolysis. This material proved to be identical to that prepared by the dihydroxylation route, confirming the stereochemical outcome of the latter process which is shown in Figure 6. Completion of aceric acid synthesis by dihydroxylation route required selective oxidation of the primary hydroxyl group which was succeffuly carried out using TEMPO-based procedure. However, purification of of carboxylic acid **20** obtained by this route proved difficult. Hence, although requiring fewer steps, the dihydroxylation route to aceric acid proved less practical than the 2-TST addition route.

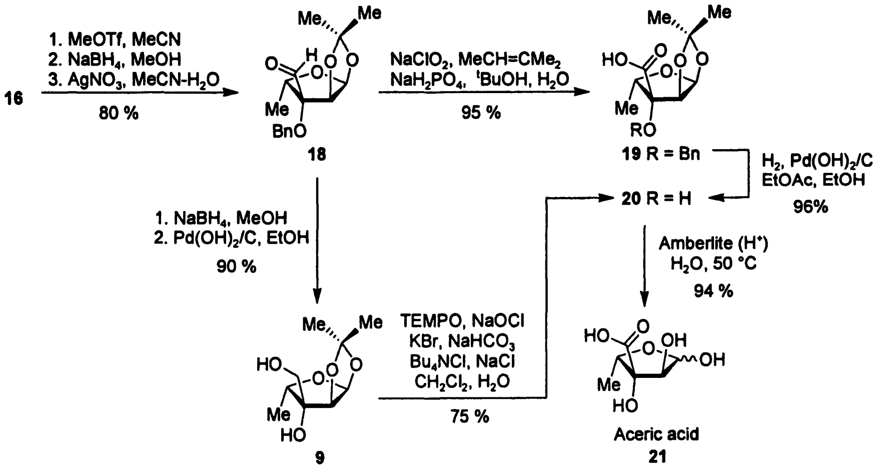

Figure 12. Transformation of 3-C-(thiazolyl)derivative 16 into aceric acid.

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Synthesis of the C-2 epimer of aceric acid.

The 3-ketofuranose derivative **22**, prepared by known methods (*34*) from L-arabinofuranose, served as a convenient starting material for introduction of the C-3 branch point *en route* to C-2 *epi*-aceric acid (Figure 13).

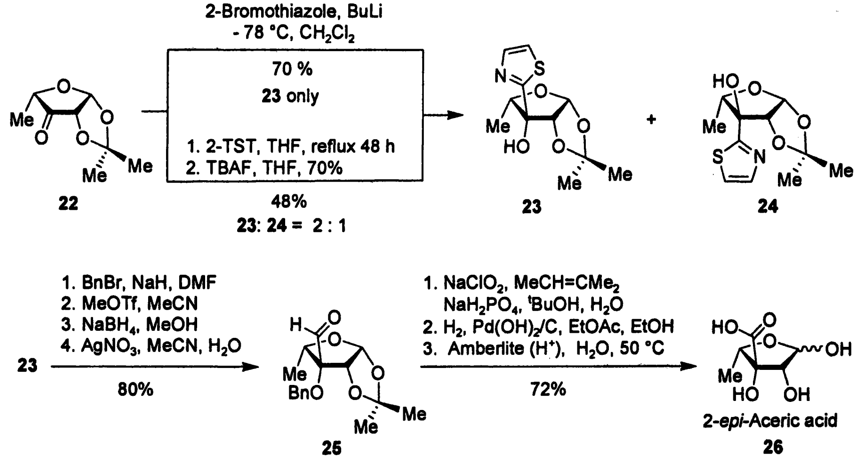
2-Bromothiazole, BuLi
- 78 °C, CH2Cl2
70 %
23 only
1. 2-TST, THF, reflux 48 h
2. TBAF, THF, 70%
48%
23: 24 = 2 : 1
22
23
24
1. BnBr, NaH, DMF
2. MeOTf, MeCN
3. NaBH4, MeOH
4. AgNO3, MeCN, H2O
80%
25
1. NaClO2, MeCH=CMe2
NaH2PO4, tBuOH, H2O
2. H2, Pd(OH)2/C, EtOAc, EtOH
3. Amberlite (H+), H2O, 50 °C
72%
2-epi-Aceric acid
26

*Figure 13. Synthesis of C-2-epi aceric acid **26**.*

Addition of 2-thiazolyl lithium to ketone **22** afforded a single diastereoisomer with the required C-3 stereochemistry, **23**, in 70% yield. The same *exo*-addition product, **23**, was identified as the major component of 2:1 mixture of stereoisomers **23** and **24** obtained in a combined 48% yield in the reaction of ketone **22** with 2-TST. In contrast to the addition of 2-TST to ketone **7**, which favours *endo*-addition (Figure 9), here the coupling yield is lower and the stereochemical preference of the reaction is for formation of *exo*-adduct **23**. Based on the limited data available (here and ref *31*), it would appear that the number of factors contribute to the stereochemical outcome of these addition reactions. *Exo*-thiazole **23** was subsequently converted to 2-*epi*-aceric acid (Figure 13) as described for conversion of **18** into aceric acid.

NMR investigations of 3-*C*-hydroxymethyl derivatives 9, 28, 29 and 30.

In addition to crystallographic data for 3-C-thiazolyl derivatives **14** and **17**, assignment of the C-3 configuration in the synthetic branched-chain sugars was accomplished using selective 1D NOE experiments. A systematic series of

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diastereomeric 3-*C*-hydroxymethyl 1,2-*O*-isopropylidene derivatives was investigated. This series included compounds **9** (Figures 6 and 12) and **28** (Figure 14) synthesized from L-xylose and their corresponding C-2 epimers **29** and **30** prepared starting from L-arabinose as shown in Figure 14.

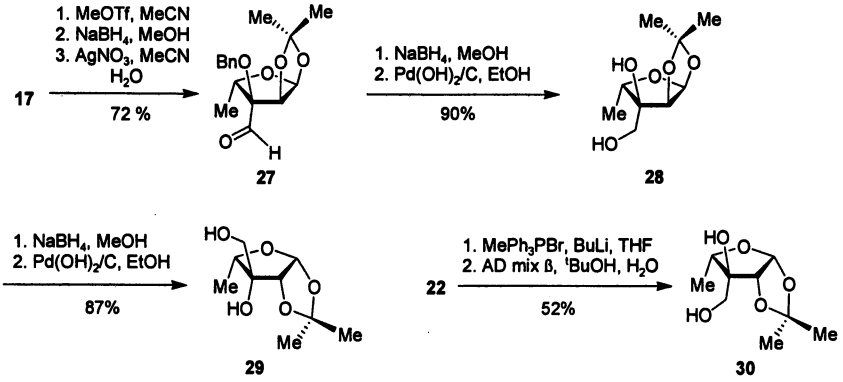

*Figure 14. Synthesis of diols **28-30**.*

The observed NOEs, which were obtained for these compounds upon selective irradiation of the signals corresponding to H-1, H-2, H-4, and H-3' protons, are summarized in Figure 15. For compounds **9** and **29** the diagnostic H-4/H-3'a and H-4/H3'b NOEs were established, indicating a 1,2-*trans*-orientation of the hydroxymethyl group and the C-5 methyl group on the furanose ring. A 1,2-*cis*-orientation of the hydroxymethyl group and the C-5 methyl group in compounds **28** and **30** was confirmed by irradiation of signals of H-3'a and H-3'b, which revealed NOEs between these protons and the H-5 protons. In addition, H-3'a/H-1 and H-3'a/H-2 NOE was detected for the diastereomer **28**. No NOE between protons of the isopropylidene group and H-3' protons was found in any of the cases studied, but strong NOE was revealed between the *exo*-methyl group of the isopropylidene moiety and H-1 and H-2 protons.

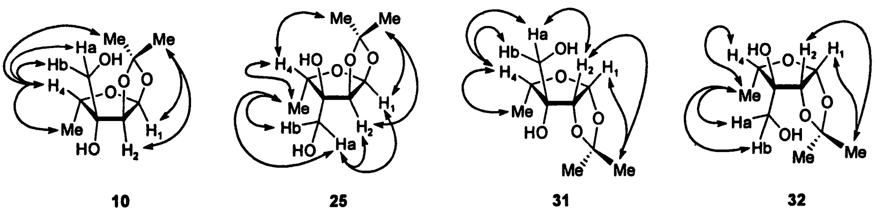

Figure 15. NOE observed for 3-C-hydroxymethyl derivatives.

Hence from this systematic NOE study it proved possible to assign C-3 stereochemistry directly by NMR spectroscopy. A key requirement to achieve this was the use of sufficiently long NMR mixing times to allow the relatively weak NOE signals evident of 5-memebred ring systems to evolve.

Summary

Syntheses of natural aceric acid have been achieved from L-xylose by dihydroxylation (10 steps, 14% yield) and trimethylsilylthiazole addition (8 steps 13% yield) routes. In addition, C-2 *epi*-aceric acid was prepared from L-arabinose (13 steps, 6% yield). The configuration of newly formed tertiary stereocentres was determined by X-ray crystallography and ^{1}H NMR spectroscopy. With a series of variously substituted derivatives in hand, studies to investigate aceric acid glycosylation chemistry are ongoing.

Acknowledgements

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Chapter 4

Iodoamidation of Glycals: A Facile Preparation of 2-Deoxy-*N*-glycosylamides

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2-Deoxy-2-iodo-N-glycosylamides have been prepared in high yields and with good selectivities by a one step process starting from glycals. The resulting *trans*-configured 2-deoxy-2-iodo-N-glycosylamides are readily converted to the 2-deoxy-N-glycosylamides by tin hydride mediated reductive deiodination. Aliphatic and aromatic primary amides, substituted ureas and amino acids were added to glycals in this fashion. The stereoselectivity and yields in these reactions were governed by a variety of factors including the nature of the solvent used in the addition reaction, the types of protecting groups on the glycal, and the nature of the R group on the amide.

Dedicated to the memory of Professor Jacques van Boom

In Frontiers in Modern Carbohydrate Chemistry; Demchenko, A.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2007.

In recent years the essential role of glycoproteins in important biological processes such as cellular recognition, adhesion, differentiation and metastasis has been recognized (*1*). Furthermore, because of the role glycoproteins play in bacterial infections, in facilitating inflammation and host immune responses to these organisms, this has lead toward important advances in the development of vaccines for the prevention of diseases (*2*).

Glycopeptides are characterized by the formation of a bond between the anomeric position of a sugar and an OH or NH group on an amino acid. Naturally-occurring O-linked glycopeptides are usually formed between a hydroxyl group of a serine or threonine residue with C1 of the sugar. N-Linked glycopeptides generally occur between the amino group of an asparagine residue and the anomeric position (*3*). In contrast to O-linked glycopeptides, N-linked glycopeptides are relatively robust to a variety of reaction conditions (e.g. acid, base) thereby making them easier to handle in synthetic transformatioms (*4*).

In recent years, even relatively simple glycosylamides have been shown to possess important biological properties. Heterocyclic glycosylamides have shown moderate activity as potential inhibitors of chitinases (*5*). N-retinoylamides have been prepared and tested as anti-tumor agents (*6*). Likewise, N-(β-D-glucopyranosyl)propanoamide was prepared as a simple mimic of the C7-C10 fragment of the anti-tumor sponge metabolites, the mycalamides (*7*). Murphy and coworkers reported that some glycosylamide analogs inhibit the binding of fibroblast growth factor (FGF-2) to heparin (*8*). Cyclodextrin polyamides and "peptidodisaccharides" have been synthesized and their binding to lectins determined (*9*).

Other uses for glycosylamides have also been described in the literature. Amphiphilic glycosylamides have been demonstrated to be a valuable class of non-ionic biosurfactants (*10*). Also, Kunz has used N-glycosylamides as an anomeric protecting group in glycosylation sequences (*4*).

Previous methods reported for the synthesis of these glycoconjugates include: 1) from glycosylamines by coupling with a carboxylic acid moiety (*11*) (most common) or by coupling with an acyl chloride or anhydride (*12*); the problem with these methods is that anomerization frequently occurs during synthesis. The coupling with acids can also be done photochemically (*13*); 2) via a Staudinger reaction with anomeric azides. Both α and β-selective ligation reactions have been realized depending on the phosphine employed (*14, 15*). With acid chlorides, triphenylphosphine can be used as a ligand. However, problems with purification of the resulting amides due to contamination with triphenylphosphine oxide can occur. To avoid this cross-contamination, polystyrene conjugated triphenylphosphine has been successfully employed in this protocol (*16*); 3) from anomeric isothiocyanates by reaction with carboxylic acids (N,N-bisglycosylthiourea formation also competes) (*17*); 4) from the Ritter

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reaction of pentenyl glycosides and carboxylic acids (*18*); and 5) by coupling of TMS-amides with anomeric sulfoxides (*19*).

All of these methods suffer from undesired side reactions, the need to do difficult purification sequences, the need to synthesize the anomeric azide or amine, low yields of product or a loss of anomeric stereocontrol in the reaction.

Reported methods for the synthesis of 2-deoxyglycosylamides are even more sparse. Although most naturally-occuring 2-deoxy-N-glycopeptides are the β-anomers, α-linked 2-deoxy-N-glycopetides have also been isolated (*20*) and this has spurred great interest in developing methods for their synthesis. For example, Thiem and coworkers reported the synthesis of an asparagines-modified-2-deoxy-α-N-glycopeptide as a potential inhibitor of the renin-angiotensin system using a 2-deoxy-2-iodo-N-succinimidylglycoconjugate (*21*). This was accomplished by ring opening of the succinimidyl moiety, peptide coupling and subsequent reductive deiodination. Recently, 2-deoxyglycosyl azides also were prepared from glycals and were used in the synthesis of 2-deoxy-β-N-glycopeptides via their corresponding glycosylamines (*22*).

We were also interested in using glycals as precursors for the synthesis of 2-deoxyglycopetides. We thought that it may also be possible to obtain the α-linked N-glycosylamide linkage directly using a variation of some chemistry first reported nearly thrity years ago by Lemieux (*23*) and Thiem (*24*); the haloglycosylation reaction..

The haloglycosylation reaction has been used extensively for the preparation of *trans*-configured 2-haloglycosides from glycals and alcohols. The greatest selectivity in addition occurs when, the bulky, participating halogen, iodine is used. The α-manno products are obtained preferentially from protected D-glucals and axial, bridging, iodonium ion intermediates have been proposed. Subsequent reductive deiodination of the 2-iodoglycosides with trialkylstannanes leads to the formation of 2-deoxy-α-glycosides .

Other anomeric functionalities have been incorporated using this approach, such as: esters (*25*), the hydroxyl group of amino acids (*26*), water (*27*), isocyanates (*28*) and isothiocyanates (*29*).

N-halosulfonamides have also been added directly to glycals (*30*). This elegant procedure afforded 2-amino-2-deoxy-β-glycosides from glycals in a two step sequence. Aziridinium ions were proposed as reactive intermediates to account for the regiochemistry and excellent stereoselectivity observed. Other weakly nucleophilic nitrogen species were aso reported to add to glycals. Thiem described the isolation of anomeric succinimides from the haloglycosylation reactions of glycals with poorly nucleophilic alcohols. Interestingly, the glycosyl imides were shown to exist preferentially in the alternate $^1C_4$ chair conformation, presumably because of the reverse anomeric effect (*31*).

These last two studies led us to investigate the possibility of using halonium ions to mediate the reactions of simple alkyl and arylamides with glycals.

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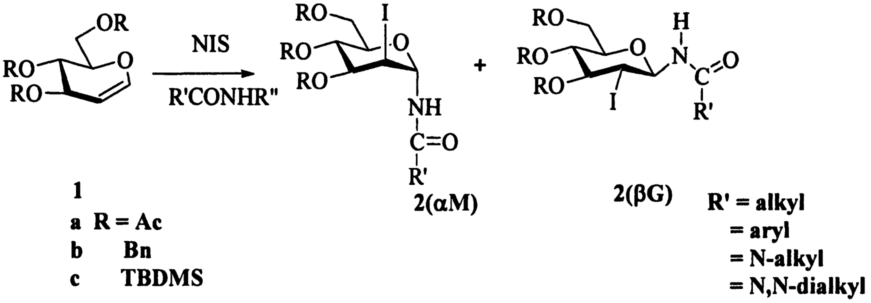
Towards this end, iodoamidation reactions were carried out on a variety of protected glucals: tri-O-acetyl-D-glucal, tri-O-benzyl-D-glucal and tris-O-*tert*-butyldimethylsilyl-D-glucal (Scheme 1, **1**) with several primary alkyl and aryl amides (*32*). The addition reactions of substituted ureas and protected amino acids were also investigated.



Scheme 1. Iodoamidation Reactions of Protected D-Glucals

As representative data in Table 1 shows, diastereomeric mixtures of gluco and manno iodoamides **2-9** were obtained from the reactions of these nitrogen nucleophiles with protected glucals. The major diastereomers observed in all cases, were the *trans* addition products, the α-manno and β-gluco isomers.

This polar addition reaction likely proceeds via the initial formation of the N-haloamide from the reaction of the amide with N-iodosuccinimide. Iodonium ion is then transferred to the glycal from the N-iodoamide and the resulting amide nitrogen attacks to effect a Markovnikov-like addition to the vinyl ether. The high facial selectivity in this reaction occurs because of : 1) the preference of the bulky halogen to add in the less sterically-hindered axial plane of the glucal and 2) the bulky, polarizable iodonium ion is capable of effectively stabilizing the developing oxocarbonium ion through a bridging species. Therefore, the nucleophilic amidic nitrogen can only then be trapped at C1 by attacking from the opposite face of the molecule.

For the addition reactions with simple amides, low temperatures were used (-78° C for 2 hr, then -78° – 25° C over 4 hr) and propionitrile was employed as the solvent (*25*). The mixtures were allowed to stir for 24-48 hr before extractive workup and subsequent column chromatography (SiO_2, gradients of hexane/ethyl acetate). Variations in the halonium species (e.g. Cl+ or Br+), the reaction solvent (diethyl ether, THF, or DMF) or the reaction temperature gave less favorable product distributions. For the simple amides, the highest yields and greatest diasteroselectivities were obtained when benzamide was used as the nucleophile (Table 1, Entries 1-3).

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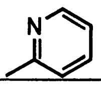
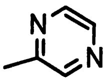
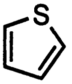
Table 1. ^{1}H-NMR Ratios[a] and Isolated Yields of Diastereomeric Iodoamides from Protected D-Glucals

Entry	Iodoamide	Sugar R =	Amide R'=	α-manno (αM)	β-gluco (βG)	Isolated yield (%)[c]
1	2a	Ac	Ph	6	10	42
2	2b	Bn	Ph	2	1	85
3	2c	TBDMS	Ph	1[b]	0	82
4	3c	TBDMS	CH_3	1 ($^1C_4$) 10 ($^4C_1$)	0	50
5	4c	TBDMS		1[b]	0	82
6	5c	TBDMS		1[b]	1	85
7	6c	TBDMS		1[b]	0	90
8	7c	TBDMS	$NHCH_2CH_3$	Nd[d]	Nd[d]	0
9	8c	TBDMS	$N(CH_3)_2$	1[b]	0	95
10	9c	TBDMS	BocGlnOMe	1[b]	0	90

[a] Integration of anomeric resonances; NMRs run in $CDCl_3$ with 0.03% added TMS

[b] Indicates only product in the $^1C_4$ conformation was observed.

[c] Purified material following column chromatography (SiO_2).

[d] No product was detected.

In the reactions with benzamide, the benzyl protected sugar afforded primarily the α-manno addition product, whereas the acetyl protected glucal gave mainly the β-gluco product. Furthermore, the electron poor acetylated glucal reacted more sluggishly than the benzyl protected precursor and gave lower yields of product. Interestingly, the *tert*-butyldimethylsilyl (TBDMS) protected glucal afforded only a single addition product in high yield when reacted with NIS and benzamide. Analysis of the proton NMR spectrum of the *tris*-tertbutyldimethylsilylated product suggested that the compound isolated from the reaction mixture existed in the alternate $^1C_4$ chair conformer (Figure 1). Similar diastereoselectivities were observed with the TBDMS- protected glucal and other primary amides. However, with acetamide a higher proportion of the alternate $^4C_1$ conformer was observed (Table 1, Entry 4).

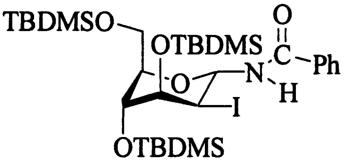
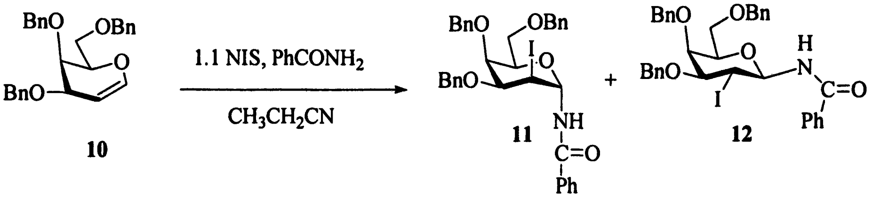


Figure 1. TBDMS-Substituted Iodobenzamides

This reaction was successful for a variety of primary amides (*31*), however, when the secondary amide, N-methylbenzamide was used in the reaction with tri-O-benzyl-D-glucal under the same conditions, none of the desired adducts were obtained. Instead, only the diequatorial succinimide adduct (70%) and starting materials were recovered.

The addition reaction also worked with other glycals. For example, reaction with tri-O-benzyl-D-galactal **10** and benzamide afforded a 2:1 ratio of diaxial:diequatorial addition products in 80% yield (Scheme 2). Based on the observed J values for the ring protons, it appeared as if there was no distortion from the normal $^4C_1$ conformation of the chair in this addition reaction.



Scheme 2. Iodoamidation Reaction of Tri-O-Benzyl-D-Galactal

The success of our methodology allowed us to pursue additional targets. The high yields and selectivities obtained with arylamides, prompted use to investigate the addition of heteroarylamides to glycals (*33*). Previously, Murphy and colleagues reported the synthesis of a series of glucuronic acid derived heteroarylglycosylamides and that many of their derivatives were capable of inhibiting the binding of heparin-albumin to Fibroblast Growth Factor (FGF-2); this receptor is believed to be important in the control of angiogenesis (*8*). Using our methodology we were also able to access a variety of analogous heteroarylglycosylamides. Several of these examples are shown in Table 1 (Entries 5-7). Because of the poor solubility of some of these amides in propionitrile, N, N-dimethylformamide was used instead as solvent in these

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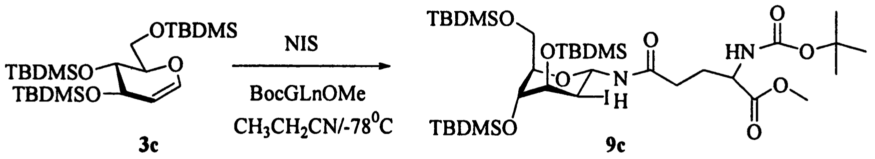
reactions. The high observed stereoselectivity in the addition reactions was unaffected by this change in solvent.

The 2-substituted heteroaryl amide, picolinamide (Table 1, entry 5), gave good yields of addition product in this reaction. Surprisingly, however, the isomeric nicotinamide (not listed), failed to react under equivalent conditions. One possible explanation for the enhanced nucleophilicity of picolinamide is the ability of the amide protons to form a hydrogen bond with the pyridine ring nitrogen. This would prevent delocalization of electrons into the carbonyl, thereby increasing the nucleophilicity of the amide nitrogen relative to its isomer. As can be seen in Table 1, the α-manno diastereomer in the alternate $^1C_4$ conformer was obtained almost exclusively from the reactions of the heteroarylamides with the TBDMS-protected glucal.

Although simple unsubstituted and monosubstituted urea derivatives (Table 1, Entry 8) failed to add to glycals using our standard reaction conditions, N,N-dimethylurea gave good yields of product (Table 1, Entry 9). Likewise, the Boc-protected methyl ester of glutamine gave good yields of the α-manno substituted 2-deoxy-2-iodoglycosylamino acid (Table 1, Entry 10) (Scheme 3).



Scheme 3. Addition of Boc-Protected Glutamine Derivative

Reductive deiodination of **2c** and **9c** was carried out using tributyltin hydride and AIBN with toluene as the solvent. The 2-deoxyglycosylamides were isolated in 79% and 89% yields, respectively, following column chromatography. The vicinal coupling constants for the ring protons on **10c** increased significantly (from 0-3.5 Hz to 6.3-7.0 Hz) for H3, H4 and H5 suggesting that the axial silyl ether groups had returned to equatorial positions about the six membered ring. Likewise for **11c**, the vicinal J-values for the ring protons were consistent with a structure more closely analogous to a $^4C_1$ chair (Scheme 4).

In conclusion, a facile, new method for the synthesis of 2-deoxy-N-glycosylamides has been developed. This method utilizes simple starting materials in a one pot protocol. The addition reaction is sensitive to electronic effects of both the sugar protecting groups and the substituents on the amide. The sterochemical outcomes and yields of the addition reaction are also dependent on the choice of solvent employed. High selectivity is achieved in the

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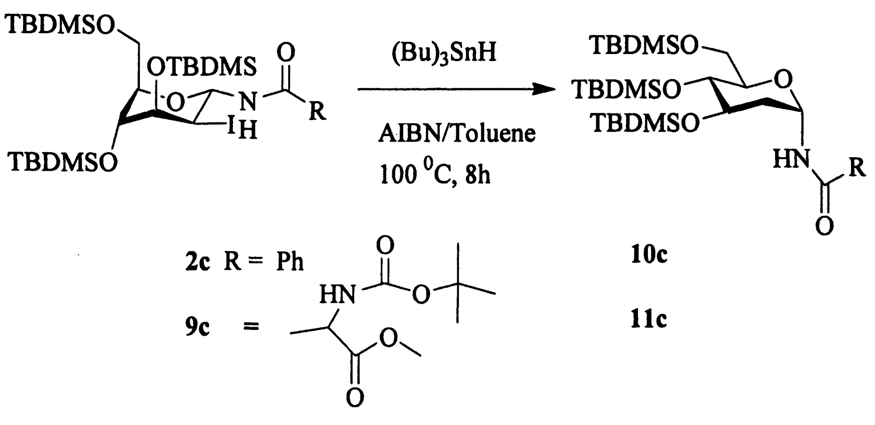

Scheme 4. Reductive Deoidination with Tin Hydride.

addition reaction, particularly when the hydroxyl groups are protected as bulky, silyl ethers. The iodoamides obtained from silylated glucals are believed to exist in the alternate $^1C_4$ chair conformation as indicated by the magnitude of the vicinal coupling constants for the sugar ring protons.

A range of primary alkyl and aryl amides have been added. In addition, substituted ureas and protected amino acids have been incorporated at C1 using this protocol. Several examples of tin hydride mediated deiodination to produce the 2-deoxyglycosylamides have been successful. Reduction of the TBDMS-protected iodoamides to the 2-deoxyglycosylamides causes a change in the sugar chair conformation as indicated by changes in the vicinal coupling constants of the ring protons.

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Chapter 5

Stereocontrolled Glycosylation: Recent Advances: β-D-Rhamnosides and β-D-Mannans

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A stereocontrolled synthesis of β-D-rhamnopyranoisdes is reported in which a modified 4,6-*O*-benzylidene group, employed to set the stereochemistry in the glycosidic bond forming step, functions as a precursor to the rhamnoside. A comparison of linear and convergent syntheses of an alternating β-(1→3)-β-(1→4)-mannan is also presented along with the development of propargyl ethers as protecting groups for improved stereoselectivity in β-mannosylation reactions.

In Frontiers in Modern Carbohydrate Chemistry; Demchenko, A.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2007.

Working initially with 4,6-*O*-benzylidene protected mannopyranosyl sulfoxides, activated with trifluoromethanesulfonic anhydride, and subsequently with the corresponding thioglycosides, and activating with the combination of 1-benzenesulfinyl piperidine and trifluoromethanesulfonic anhydride, we have developed a facile, highly stereocontrolled synthesis of the β-mannopyranosidic linkage (*1-4*). The key to success in this chemistry is the presence of the 4,6-*O*-benzylidene acetal which serves to destabilize the mannosyl oxacarbenium ion relative to the covalent α-mannosyl triflate intermediate, thereby ensuring rapid β-selective quenching of a transient contact ion pair by the incoming glycosyl acceptor (Fig. 1) (*5, 6*).

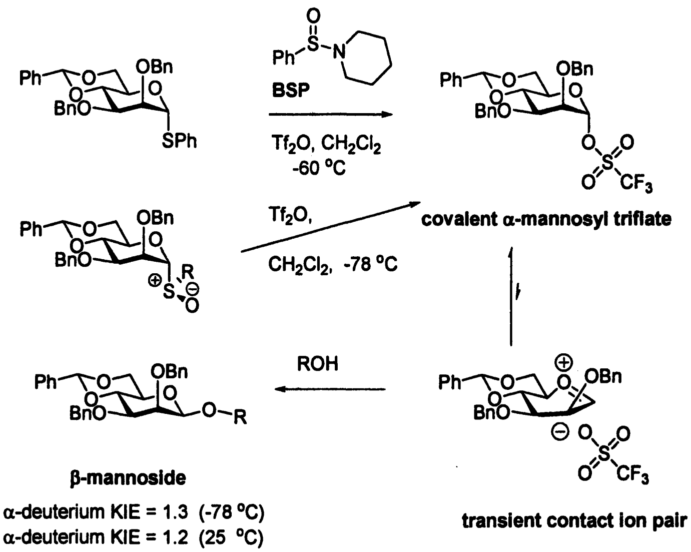
BSP
Tf$_2$O, CH$_2$Cl$_2$
-60 °C
Tf$_2$O,
CH$_2$Cl$_2$, -78 °C
covalent α-mannosyl triflate
ROH
β-mannoside
α-deuterium KIE = 1.3 (-78 °C)
α-deuterium KIE = 1.2 (25 °C)
transient contact ion pair

Figure 1. The β-mannosylation reaction

Having completed numerous syntheses of β-mannoside containing oligosaccharides and other glycoconjugates using this methodology (*7-10*), we turned out attention to the β-L-rhamnopyranosides (*11-13*), and D-rhamnopyranosides. This type of linkage, which occurs widely in Nature, presents the same stereochemical challenge as the β-mannosides but raises the bar to a higher level by virtue of the 6-deoxy functionality. We hypothesized that the β-D-rhamnopyranosides might be accessed via the 4,6-*O*-benzylidene protected β-mannosylation, followed by a Hanessian-Hullar type fragmentation of the benzylidene acetal to the 6-bromo-4-*O*-benzoate-β-D-rhamnoside (*14-18*), with a final reductive debromination providing the target structure. Alternatively, the purely radical fragmentation of 4,6-*O*-benzylidene acetals,

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involving simple heating with a thiol and a radical initiator (*19-21*) could take us directly from a benzylidene protected mannoside to a 4-*O*-benzoyl rhamnoside in a single step, avoiding the 6-bromo intermediate. Unfortunately, despite isolated reports the literature to the contrary, neither of these chemistries are compatible with the presence of benzyl ethers in our hands. We note, however, a very interesting large-scale NBS cleavage of a benzylidene acetal of a 1,2-diol in the presence of multiple benzyl ethers which, presumably, is possible because of the greater lability of dioxolane C-H bonds as compared to those in dioxanes (*22*). We turned therefore to alternative sources of the benzylidene radical, compatible with both the **BSP** activation protocol and the presence of multiple benzyl ethers. A system was designed based the tributyltin hydride mediated generation of acyl radicals from 2-(2-iodophenyl)ethyl thiolesters (*23*) and decarbonylation such as we had previously employed in our laboratory to obtain nucleotide C4' radicals (*24, 25*) After a control experiment to establish the stability of the thiolester function to the **BSP**/trifluoromethanesulfonic anhydride glycosylation conditions, diol **1** was converted to the acetal **2** by means of a transacetalization reaction. Conversion of the methyl ester to the requiste thiol ester **3** then was achieved with 2-(2-iodophenyl)ethanethiol and trimethylaluminum. A series of coupling reactions were then conducted to a range of standard primary and secondary carbohydrate alcohols as well as to 1-adamantanol. In each case exquisite selectivity for the β-anomer **4** was obtained, independent of the isomer employed at the remote benzylidene stereogenic center. Finally, a cascade of radical reactions was initiated by exposure of the glycosides to tributyltin hydride and a radical initiator in benzene at reflux, resulting in the formation of the 6-deoxy-β-D-mannosides **5**, or β-D-rhamnosides, in high yield (Figures 2 and 3) (*26*). As anticipated on the basis of the precedent from Roberts' work (*20, 27*) these benzylidene radical fragmentations were highly regioselective for formation the 6-deoxy in preference to the 4-deoxy system.

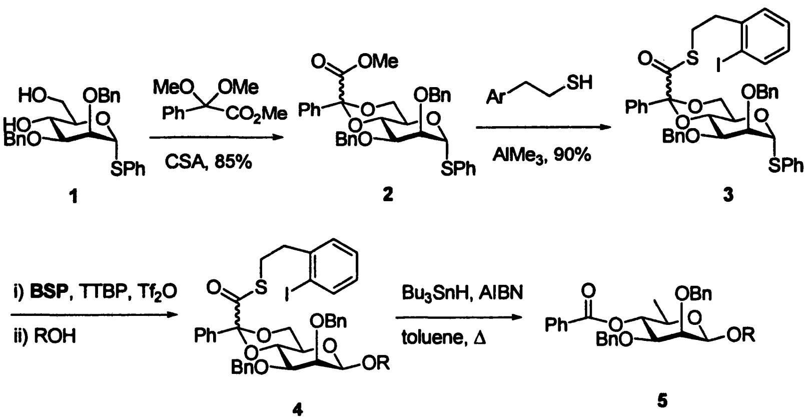

Figure 2. Stereocontrolled Synthesis of β-D-Rhamnosides

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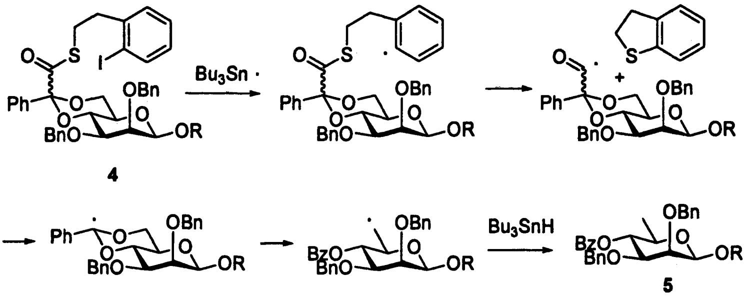

Figure 3. Mechanism of the Radical Fragmentation Reaction

With a suitable method established, attention was turned to the tetrasaccharide repeating unit of the lipopolysaccharide from *Escherichia hermannii* ATCC33650 and 33652, which is characterized by an unusual juxtaposition of α- and β-D-rhamnopyranosides (*28*). Earlier work from our laboratory had revealed 4,6-*O*-benzylidene protected mannosyl donors bearing a carboxylate ester at O-3 to be very highly α-selective (*29*), thereby setting the stage for the synthesis of the two D-rhamnoside units in a single step. The synthesis (*8*) began with the β-mannosyl donor **6** which was coupled to methyl 2,3,6-tri-*O*-benzyl-β-D-glucopyranoside under the standard BSP/trifluoromethanesulfonic anhydride conditions in the presence of 2,4,6-tri-*tert*-butylpyrimidine, our preferred non-nucleophilic base (*30*) resulting in the formation of the disaccharide **7** in 84% yield. The naphthylmethyl protecting group was then selectively removed with DDQ giving alcohol **8** ready for coupling to the α-mannosyl donor **9**. Donor **9**, which was obtained from **6** by removal of the naphthylmethyl group followed by chloroacetylation, was not activated by the **BSP/**Tf_2O activation protocol and necessitated the apparently more powerful diphenyl sulfoxide/Tf_2O combination introduced by the van Boom group (*31*). Addition of alcohol **8** to the activated **9** provided the expected trisaccharide **10** in 76% yield as a pure α-anomer. The chloroacetate protecting group was then removed selectively in the presence of the two sterically hindered thiol esters with ethylene diamine in methanol (Figure 4).

Trisaccharide **11** was then exposed to tributyltin hydride and AIBN in toluene at reflux resulting in a double cascade of radical reactions and providing trisaccharide **12** containing both the α- and β-D-rhamnopyranosides in 55% yield (Figure 5) in the presence of five benzyl ethers. Subsequent galactosylation with a 4,6-*O*-benzylidene protected galactosyl donor provided the tetrasaccharide **13** in 64% yield and setting the stage for the final deprotection by saponification, then hydrogenolysis.

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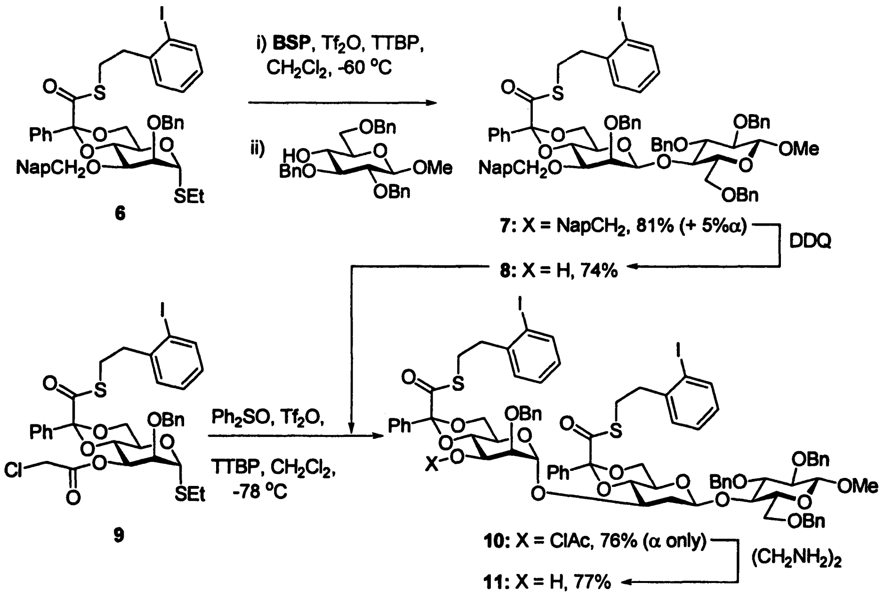
6
i) BSP, Tf_2O, TTBP, CH_2Cl_2, -60 °C
ii)
7: X = $NapCH_2$, 81% (+ 5%α)
DDQ
8: X = H, 74%
9
Ph_2SO, Tf_2O, TTBP, CH_2Cl_2, -78 °C
10: X = ClAc, 76% (α only)
$(CH_2NH_2)_2$
11: X = H, 77%

Figure 4. Assembly of a Trisaccharide

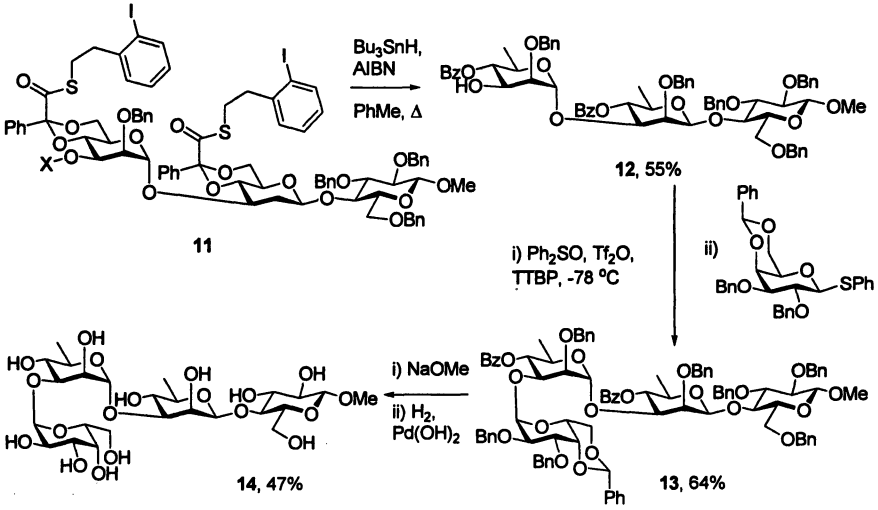
11
Bu_3SnH, AIBN
PhMe, Δ
12, 55%
i) Ph_2SO, Tf_2O, TTBP, -78 °C
ii)
13, 64%
i) NaOMe
ii) H_2, $Pd(OH)_2$
14, 47%

Figure 5. Double Radical Fragmentation and Completion of the Synthesis

We have also been interested in the stereocontrolled synthesis of β-mannans and have previously described linear syntheses of β-(1→2)-, β-(1→4)-, and an alternating β-(1→3)-β-(1→4)-mannan employing the 4,6-*O*-benzylidene directed β-mannosylation (*9, 10, 32*). The linear synthesis of a hexasaccharide subunit of the β-(1→3)-β-(1→4)-mannan common to *Rhodotorula glutinis, Rhodotorula mucilaginosa* and *Leptospira biflexa*, in which all couplings proceeded in high yield and selectivity, is illustrated in Figure 6 (*32*).

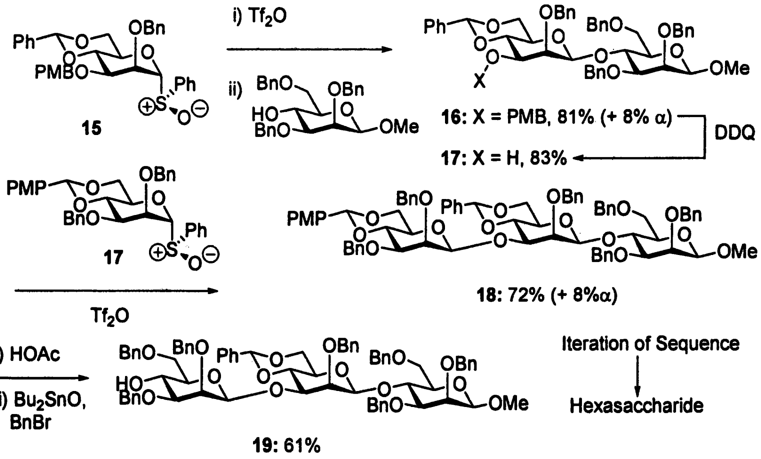

Figure 6. Linear Synthesis of a β-(1→3)-β-(1→4)-Mannan

To improve the overall efficiency of our syntheses we investigated a convergent block synthesis of the same hexasaccharide **19** (*10*). The synthesis of a trisaccharide donor **26** representing the left-hand side of the target proceeded uneventfully with both couplings giving the familiar high β-selectivity (Figure 7). The only noteworthy point in this sequence is the application of glycosyl acceptors **21** and **24** which are themselves thioglycosides. No differentiation between thioglycosides based on the use of arming and disarming protecting groups, or of more or less electron rich thioglycosides, is required when the donor is converted to the glycosyl triflate prior to addition of the acceptor as is our practice (Figure 1). Nevertheless, following the suggestion of van Boom, triethylphosphite was added to the reaction mixture after the acceptor to minimize damage to the remaining thioglycoside by extraneous thiophiles (*31, 33*).

The synthesis of a second trisaccharide, representing the right-hand side of the target proved more interesting, especially as it included a rare example of a

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4,6-*O*-benzylidene directed β-mannosylation that proceeded in high yield but with poor selectivity of approximately 1:1 (Figure 8). It is noteworthy that disaccharide **22** was featured in the syntheses of both trisaccharides **26** and **28**, thereby increasing the efficiency of the overall process.

The two trisaccharides were next coupled under the standard **BSP** conditions to give the β-(1→3)-β-(1→4)-mannan **29** in 35% yield together with

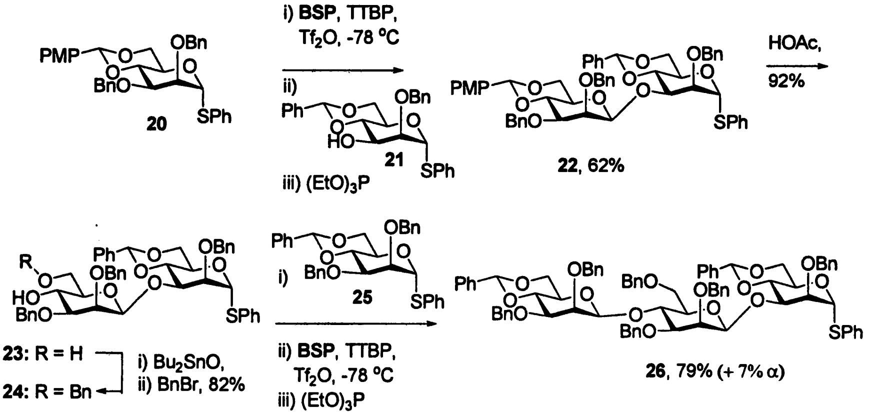
PMP
OBn
BnO
SPh
20
i) BSP, TTBP, Tf_2O, -78 °C
ii)
Ph
HO
21
iii) $(EtO)_3P$
22, 62%
HOAc, 92%
R
23: R = H
24: R = Bn
i) Bu_2SnO, ii) BnBr, 82%
i)
25
ii) BSP, TTBP, Tf_2O, -78 °C
iii) $(EtO)_3P$
26, 79% (+ 7% α)

Figure 7. Synthesis of a Trisaccharide Donor

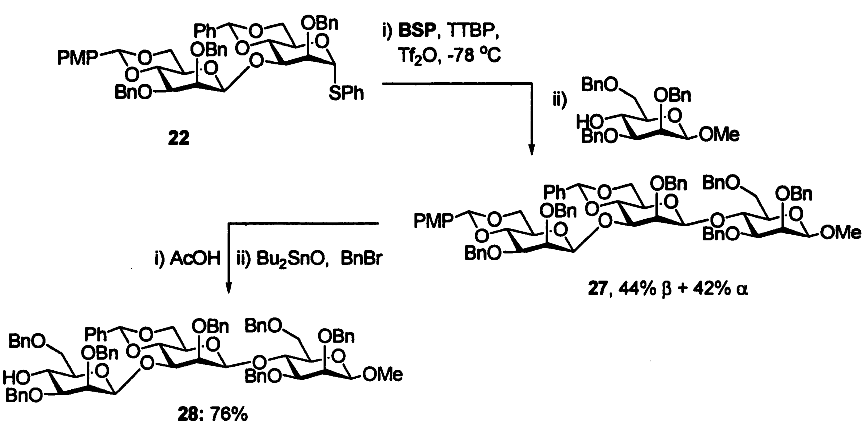
PMP
Ph
OBn
BnO
SPh
22
i) BSP, TTBP, Tf_2O, -78 °C
ii)
HO
OMe
27, 44% β + 42% α
i) AcOH
ii) Bu_2SnO, BnBr
28: 76%

Figure 8. Synthesis of a Trisaccharide Acceptor

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53% of the α-anomer at the newly created linkage (Figure 9). The two anomers were readily separated and the all β-one was found to be identical to the sample obtained from the linear route (Figure 6). Global deprotection of **30** was achieved by hydrogenolysis over palladium charcoal giving, finally, the β-mannan **30** (*10*).

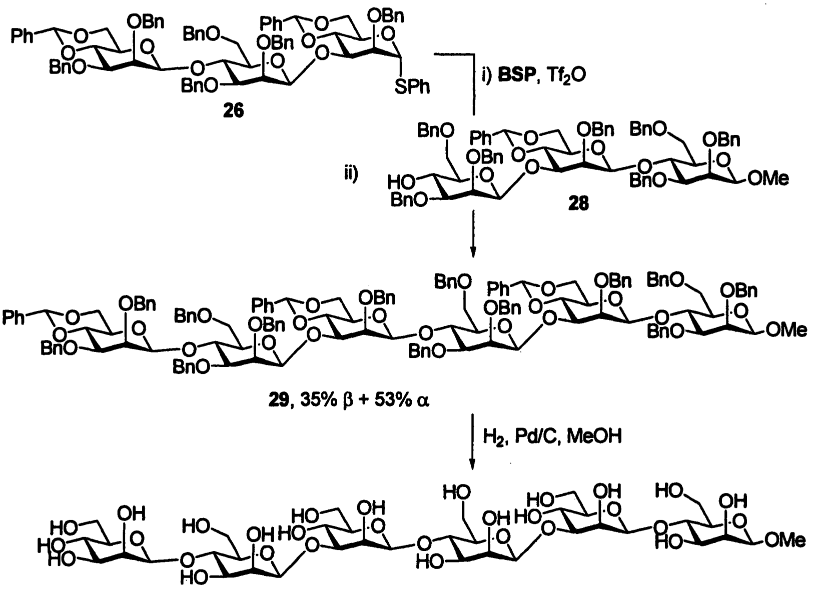

Figure 9. Convergent Synthesis of of a β-(1→3)-β-(1→4)-Mannan

In spite of the very poor selectivity in two of the key coupling reactions, comparison of the two routes to **29** revealed the convergent one to be the more efficient of the two syntheses with 8 steps and 4.4% overall yield, excluding synthesis of the monosaccharide building blocks, as compared to the 11 steps and 1.9% overall yield of the linear route.

The two poorly selective coupling steps en route to **29** by the convergent route have in common glycosyl donors **22** and **26** carrying bulky glycosidic units on O-3. In this they resemble an earlier instance of unselective β-mannosylation in which the 2-*O*-benzyl-3-*O*-silyl donor **31** gave much poorer selectivity than the corresponding 3-*O*-benzyl-2-*O*-silyl regioisomer **32** (Figure 10) (*34*).

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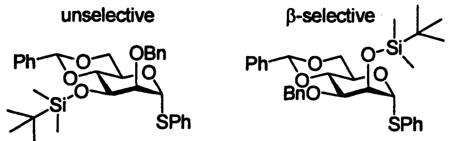

31 32

Figure 10. Selective and Unselective Regioisomeric Mannosyl Donors

We reasoned that donors **22**, **26**, and **31** suffer from a common buttressing problem in which the bulky group on O-3 restricts the conformational space available to the protecting group on O-2. This effectively forces the O-2 protecting group to spend more time in the proximity of the anomeric center thereby causing abnormal shielding of the β-face and leading to reduced selectivity. While the problem can obviously be overcome for the silylated donor **31** by using a smaller group on O-3, as was the case in our synthesis of the common core trisaccharide of the *N*-glycoproteins (*35*), an alternative solution must be found in the case of donors such as **22** and **26**. We reasoned that both the buttressing effect and the resulting hindrance of the anomeric center would be minimized by employing the smallest possible O-2 protecting group consistent with subsequent deprotection. To this end, working first with the readily accessible 3-*O*-silyl system, we prepared the 2-*O*-allyl and 2-*O*-propargyl donors **33** and **34** and coupled them with a glycosyl acceptor under the standard **BSP** conditions (Figure 11). While both donors gave excellent yields of disaccharides, the stereoselectivities differed considerably. The 2-*O*-allyl donor **33** gave the disaccharide **35** in 90% yield but with only 1.5:1 β:α selectivity, reminiscent of the 2-*O*-benzyl donor **31**. In contrast, the 2-*O*-propargyl donor **33** showed considerably improved selectivity in the formation of **36** with a β:α ratio of 5:1 and an 89% isolated yield. The propargyl ether was therefore selected for further development (*36*).

With enhanced selectivity in hand, we turned to deprotection before applying the new group in oligosaccharide synthesis. In the event, this was readily realized by isomerization to the allenyl ether with potassium *tert*-butoxide and then exposure to catalytic osmium tetroxide and *N*-methylmorpholine *N*-oxide (Figure 12). Although, apparently not used as protecting previously alternative methods for the cleavage of propargyl ethers have been reported (*37, 38*).

Finally, the synthesis of a short segment of a β-(1→3)-linked mannan was undertaken to test the viability of the new protecting group in the context of convergent oligosaccharide synthesis. Thus, donor **25** was coupled to acceptor **38** in the standard manner, with the addition of triethyl phosphite to protect the

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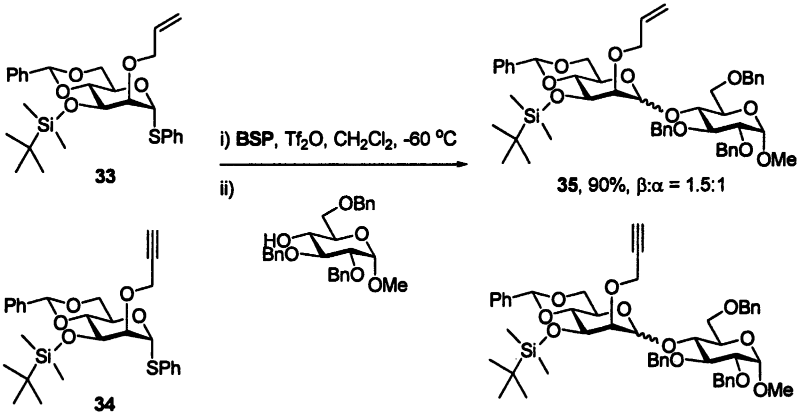
Ph
Si
O
SPh
33
34
i) BSP, Tf$_2$O, CH$_2$Cl$_2$, -60 °C
ii)
HO
BnO
OBn
OMe
35, 90%, β:α = 1.5:1
36, 89%, β:α = 5:1

Figure 11. 2-O-Allyl and Propargyl-3-O-Silyl Mannosyl Donors

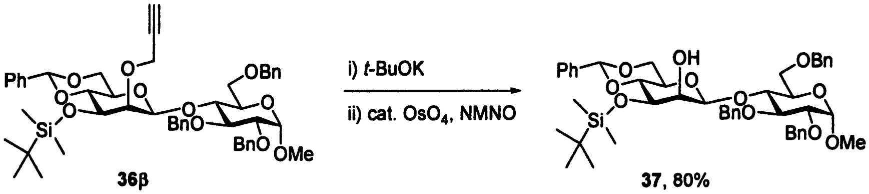
Ph
Si
OBn
BnO
OMe
36β
i) t-BuOK
ii) cat. OsO$_4$, NMNO
OH
37, 80%

Figure 12. Selective Propargyl Ether Cleavage

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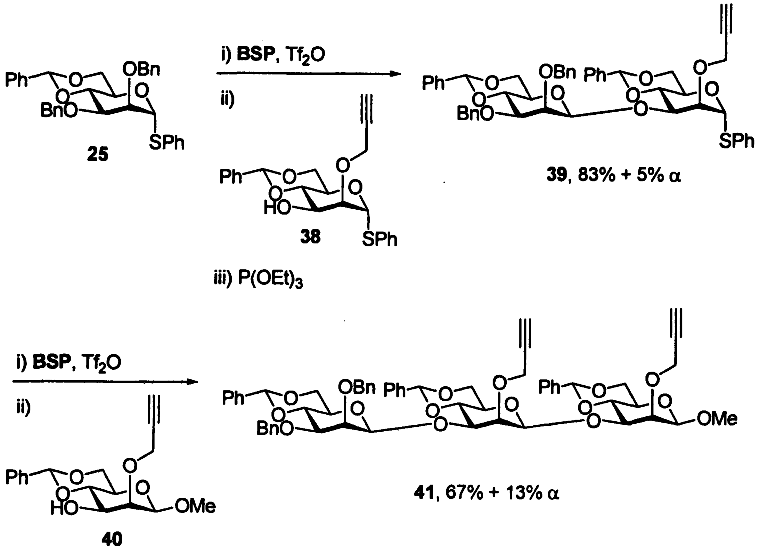
i) BSP, Tf2O
ii)
iii) P(OEt)3
25
38
39, 83% + 5% α
i) BSP, Tf2O
ii)
40
41, 67% + 13% α
Ph
OBn
BnO
SPh
HO
OMe

Figure 13. Synthesis of a β-(1→3)-Linked Mannan Segment

newly introduced thioglycoside from premature activation, giving the disaccharide donor **39** in 83% yield along with 5% of the α-anomer (Figure 13). This donor was then coupled to the acceptor **40** in the standard manner giving the all β-trisaccharide **41** in 67% yield along with 13% of the α-anomer. This represents a β:α selecivity of 5:1 which is to be contrasted with the much poorer selectivity of 1:1 obtained with the disaccharide donor **22** in Figure 8.

Finally, both propargyl groups were selectively removed from **41** by the base catalyzed isomerization, catalytic osmoylation protocol (Figure 14).

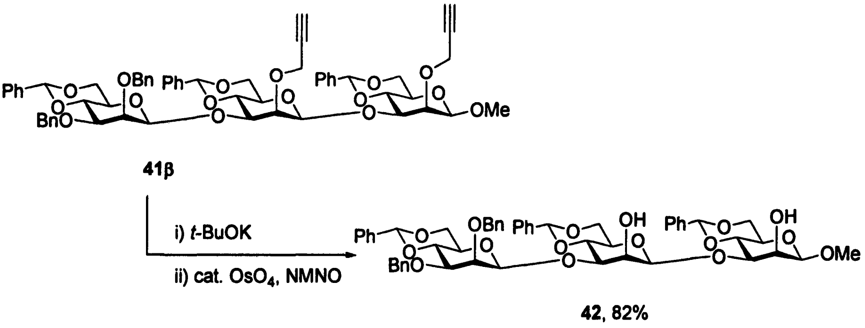
41β
i) t-BuOK
ii) cat. OsO4, NMNO
42, 82%
Ph
OBn
BnO
OH
OMe

Figure 14. Concomitant Removal of Two Propargyl Ethers

In summary, the judicious design and application of new protecting groups has enabled the range of glycosidic bonds accessible stereoselectively by the thioglycoside/**BSP**/Tf_2O or the closely related thioglycoside/Ph_2SO/Tf_2O methodology to be extended to the β-D-rhamnopyranosides and to the convergent synthesis of β-(1→3)-linked mannans.

Acknowledgments

The work reported in this chapter was made possible by the skillful and insightful contributions of Qingjia Yao, Hongmei Li, Wenju Li, and Prasanna Jayalath, as well as by the financial support of the National Institutes of Health (GM 57335) to whom we are especially grateful.

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Chapter 6

Stereoselective Glycosylations Using Chiral Auxiliaries

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The stereoselective introduction of a glycosidic bond presents the greatest challenge to complex oligosaccharide synthesis. Important developments such as automated polymer supported oligosaccharide synthesis will not realize their full potential until this problem is addressed. We have developed a novel approach for stereoselective glycosylations whereby a chiral auxiliary at C-2 of a glycosyl donor controls the anomeric outcome of a glycosylation.

Introduction

Glycoconjugates are the most functionally and structurally diverse compounds in Nature and it is now well established that protein- and lipid-bound saccharides play essential roles in many molecular processes impacting eukaryotic biology and disease.[1-3] Examples of such processes include fertilization, embryogenesis, neuronal development, hormone activities, the proliferation of cells and their organization into specific tissues. Remarkable changes in the cell-surface carbohydrates occur with tumor progression, which

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ACS Symposium Series; American Chemical Society: Washington, DC, 2007.

appears to be intimately associated with the dreaded state of metastasis. Furthermore, carbohydrates are capable of inducing a protective antibody response, which is a major contributor to the survival of an organism during infection. Oligosaccharides have also been found to control the development and defense mechanisms of plants. The increased appreciation of the role of carbohydrates in the biological and pharmaceutical sciences has resulted in a revival of interest in carbohydrate chemistry.

A major obstacle to advances in glycobiology is the lack of pure and structurally well-defined carbohydrates and glycoconjugates. These compounds are often found in low concentrations and in microheterogeneous forms, greatly complicating their isolation and characterization. In many cases, well-defined oligosaccharides can only be obtained by chemical- or enzymatic synthesis. Although these approaches for obtaining complex oligosaccharides are plagued with problems, synthetic compounds are increasingly used to address important problems in glycobiology research and for vaccine and drug discovery.

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Recent Progress in the Synthesis of Complex Oligosaccharides

Despite complex oligosaccharides synthesis is plagued by problems, significant improvements have been made during the past decade.[4-16] New leaving groups for the anomeric center have been developed, which can be introduced under mild reaction conditions and are sufficiently stable for purification and storage for a considerable period of time. The most commonly employed glycosyl donors include anomeric fluorides, trichloroacetimidates, and thioglycosides. The glycal assembly strategy,[17] the use of anomeric sulfoxides[18] and dehydrative glycosylation protocols are also emerging as attractive tools for the assembly of complex oligosaccharides. Convergent synthetic strategies that allow the convenient assembly of complex oligosaccharides from properly protected building units involving a minimum number of synthetic steps have become available. Methods for solid phase oligosaccharide synthesis have been reported and these procedures shorten oligosaccharide synthesis by removing the need to purify intermediate derivatives.[15] Recently, automated solid-phase synthesis was used for the preparation a branched dodecasaccharide. For this purpose, an automated peptide synthesizer was re-engineered to allow repetitive chemical manipulations at variable temperatures.[16] An acceptor substrate was attached to a 1% cross-linked polystyrene resin that was modified by an octenediol linker. Trichloroacetimidates were employed as glycosyl donors in combination with acetyl esters as temporary protecting groups. At the end of the coupling cycles, the linker could easily be cleaved by an olefin cross metathesis to give a protected saccharide as a pentenyl glycoside. The need for increasingly efficient methods for oligosaccharide synthesis has stimulated the development of enzymatic methods.[4-9] These enzymatic methods bypass the need for protecting groups since the enzymes control both the regio- and stereoselectivity of glycosylation.

The Problem of Anomeric Control in Glycosylations

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The stereoselective introduction of a glycosidic linkage is one of the most challenging aspects in complex oligosaccharide synthesis. The nature of the protecting group at C-2 of a glycosyl donor is a major determinant of the anomeric selectivity. A protecting group at C-2, which can perform neighboring group participation during a glycosylation, will give 1,2-*trans* glycosides.[19,20] On the other hand, 1,2-*cis* glycosides can be obtained when a non-assisting functionality is present at C-2. In these glycosylations, the reaction conditions (*e.g.* solvent, temperature, and promoter) as well as the constitution of the glycosyl donor and acceptor (*e.g.* type of saccharide, leaving group at the anomeric center, protection and substitution pattern) will determine the anomeric selectivity.[21] In general, efforts to introduce 1,2-*cis* glycosides lead to mixtures of anomers. Separation of these anomers requires time-consuming purification protocols resulting in loss of material. It also limits the use of one-pot multi-step glycosylations[22,23] and automated polymer-supported synthesis.[15,24,25] The stereoselective formation of 1,2-*cis* glycosides is one of the principal challenges of complex oligosaccharide synthesis. The next section will provide a brief overview of the most important methods for controlling anomeric selectivity in glycosylations.

The most reliable method for the introduction of 1,2-*trans* glycosidic linkages is based on neighboring group participation of a 2-*O*-acyl functionality. In these glycosylations, a promoter (A) activates an anomeric-leaving group, which results in its departure and the formation of an oxacarbenium ion. Subsequent, neighboring group participation by a 2-*O*-acyl protecting group leads to the formation of a more stable dioxolenium-ion. Attack of an alcohol at the anomeric centre of the dioxolenium-ion results in the formation of a 1,2-*trans* glycoside (Scheme 1a). Thus, in the case of glucosyl-type donors, β-linked products will be obtained while mannosides will give α-glycosides. The neighboring group assisted glycosylation procedures are compatible with many different glycosylation protocols and anomeric leaving groups. In some glycosylations, the alcohol may attack at the C-2 position of the dioxolane-ring of the dioxolenium-ion, resulting in the formation of an undesired ortho-ester. Ortho-ester formation may be prevented by the use of a C-2 benzoyl- or pivaloyl group. In some cases, the glycosylation may also proceed *via* the oxacarbenium ion leading to mixtures of anomers.

1,2-*cis*-Glycosides can be synthesized when a non-assisting functionality is present at C-2 of a glycosyl donor. In general, these glycosylations require optimization of reaction conditions such as solvent, temperature, and promoter to achieve acceptable anomeric selectivities. The first procedure for the selective formation of 1,2-*cis* glycosides such as α-glucosides and α-galactosides was introduced by Lemieux and coined 'the *in-situ* anomerization procedure' (Scheme 1b).[26] In this type of glycosylation, an activator catalyzes an equilibration between an α- and β-halide. This equilibrium is shifted strongly towards the α-halide since this compound is stabilized by an endo-anomeric effect. However, the energy barrier for nucleophilic attack by an alcohol is lower for the β-halide leading to the formation of an α-glycoside. An important requirement of this reaction is that the rate of equilibration is much faster than that of glycosylation. High α-anomeric selectivities have been obtained with other anomeric leaving groups. Although the reaction mechanisms of these glycosylations have not been studied in detail, it is reasonable to assume that they proceed *via* an *in-situ* anomerization process and probably α- and β-ion pairs are formed as reactive intermediates.

β-Linked mannosides are another class of *cis*-glycosides that are difficult to introduce in a stereoselective manner. These glycosidic linkages have been introduced by the activation of α-halides with insoluble silver salts (Scheme 1c).[7,27] In this case, anomerization of the halide is restricted because of the absence of nucleophiles in the reaction mixture. Therefore, these glycosylations proceed with inversion of configuration. Silver silicate and silver silicate-aluminate have been applied as heterogeneous catalyst. Recently, β-mannosides have been prepared in a highly stereoselective manner by an intramolecular aglycon delivery approach (Scheme 1d).[28-31] In this approach, the sugar alcohol (ROH) is first linked *via* an acetal or silicon tether (Y = CH_2, methoxybenzylidene or $SiMe_2$) to the C-2 position of a mannosyl donor. Subsequent activation of the anomeric centre of this adduct forces the aglycon to be delivered from the β-face of the glycosyl donor. The research group of Crich has pioneered an attractive approach for the introduction of β-mannosides by the *in-situ* formation of an anomeric triflate (Scheme 1e).[32-36] This triflate is only formed as an α-anomer because of a strong endo-anomeric effect. An S_N2 like-displacement of the α-triflate by a sugar hydroxyl will result in the formation of a β-mannoside. A prerequisite of β-mannoside formation is that the mannosyl donor is protected by a 4,6-*O*-benzylidene acetal, which opposes oxacarbenium because of torsional strain engendered on going to the sofa conformation of this intermediate. This method has been extended to the introduction of β-rhamnosides.[37,38]

A participating solvent can also control the stereochemical outcome of a glycosylation. A marked example is the use of acetonitrile,[6] which in many cases leads to the formation of mainly equatorial glycosidic bonds (*e.g.* β-glucosides and β-galactosides). Several groups have independently proposed that this

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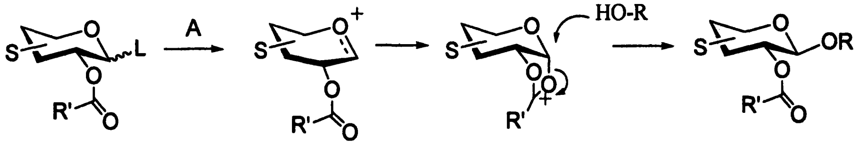
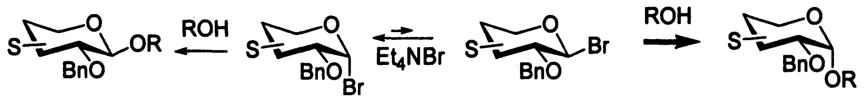
reaction proceeds *via* an α-nitrilium ion. Nucleophilic substitution of the nitrilium ion by an alcohol will lead to mainly, but not exclusively, β-glycosides (Scheme 1f). The use of diethyl ether as a solvent is known to increase the α-anomeric selectivity of glycosylations.[39]

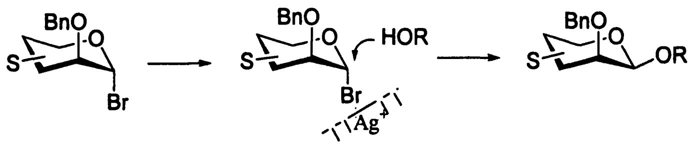
(a) Preparation of 1,2-*trans* glycosides by neighbouring group participation


S
O
L
R'
A
+
HO-R
OR

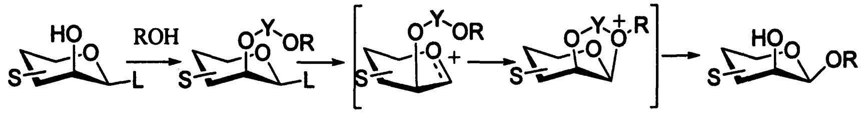
(b) Preparation of α-glycosides by *in situ* anomerization


S
O
OR
BnO
ROH
Br
Et$_4$NBr
ROH

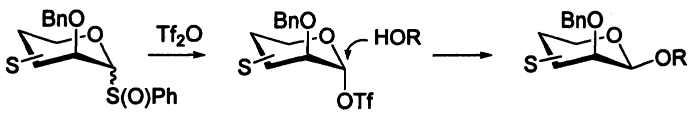
(c) Preparation of β-mannosides by using insoluble silver salts


BnO
S
O
Br
HOR
Ag
OR

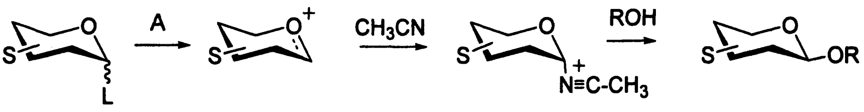
(d) Synthesis of β-mannosides by intra molecular aglycon delivery


HO
S
O
L
ROH
Y
OR
+
R

(e) Introduction of β-mannosidic linkage by *in-situ* formation of an α-triflate


BnO
S
O
S(O)Ph
Tf$_2$O
HOR
OTf
OR

(f) Preparation of β-glycosides by participation of the solvent acetonitrile


S
O
L
A
+
CH$_3$CN
N≡C-CH$_3$
ROH
OR

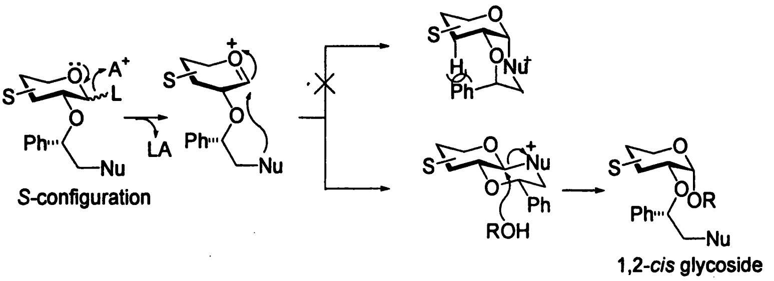
Scheme 1. Methods for the stereoselective formation of glycosidic linkages.

Stereoselective Glycosylations Using Chiral Auxiliaries

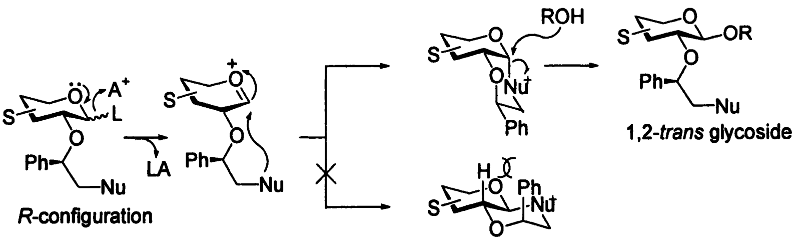
We have developed a novel approach for stereoselective glycosylations using a chiral auxiliary at C-2 of a glycosyl donor (Schemes 2a, b).[40] The auxiliary is a C-1 substituted ethyl moiety that contains a nucleophilic group (Nu). Upon formation of an oxacarbenium ion, the nucleophilic moiety of the auxiliary will participate, leading to the formation of either a *trans*- or *cis*-decalin system. It is expected that an auxiliary with *S*-stereochemistry will lead only to the formation of *trans*-decalin since the alternate *cis*-fused system will place the phenyl-substituent in an axial position inducing unfavorable steric interactions (Scheme 2a). Subsequent displacement of the anomeric moiety of the *trans*-decalin intermediate will lead to the formation of a 1,2-*cis* glycoside. Alternatively, the use of an auxiliary with *R*-stereochemisty will lead to the formation of a 1,2-*trans*-glycoside because in this case the *trans*-decalin system will experience unfavorable steric interactions. Therefore glycosylation will only take place from the *cis*-decalin intermediate (Scheme 2b).

Ethyl mandelate was explored as a first generation chiral auxiliary because both enantiomers of this compound are readily available. Furthermore, esters are

(a) Neighboring group participation by an *S* auxiliary at C-2 leading to 1,2-*cis* glycosides



(b) Neighboring group participation by an *R* auxiliary at C-2 leading to 1,2-*trans* glycosides



A = activator, Nu = nucleophile, L = leaving group

Scheme 2. New approaches for stereoselective glycosylation

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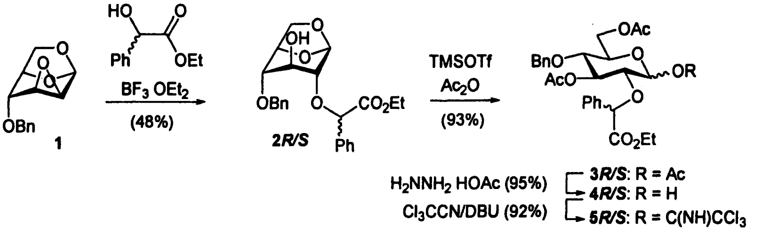
well-established as appropriate participating functionalities in glycosylations and the benzylic nature of the auxiliary will make it possible to remove it under reductive conditions. Glucosyl donors **5R** and **5S**, containing a (*R*)- or (*S*)-ethoxycarbonylbenzyl moiety, could be prepared by an efficient procedure starting from the readily available epoxide **1**.[41] Thus, reaction of **1** with ethyl *R*-mandelate in the presence of BF_3-OEt_2 led to a *trans*-diaxial opening of the epoxide to give **2R** in a yield of 48%. Next, acetolysis of the 1,6-anhydro-bridge of **2R** with acetic anhydride and catalytic amount of TMSOTf gave compound **3R** in an almost quantitative yield. The anomeric acetyl group of **3R** could be selectively removed with hydrazinium acetate to give hemiacetal **4R**, which could be converted into trichloroacetimidate **5R** using trichloroacetonitrile in the presence of DBU.[8] Glycosyl donor **5S** could be prepared by a similar protocol using ethyl *S*-mandelate as the starting material (Scheme 3).



Bn = benzyl, DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene, TMSOTf = trimethylsilyl trifluoromethanesulfonate.

Scheme 3. Preparation of glycosyl donors 5R/S

Coupling of **5S** with **9** using a catalytic amount of TMSOTf in dichloromethane at –78°C gave disaccharide **14S** mainly as the α-glycoside in an almost quantitative yield. As expected, coupling of **5R** with **9** under similar reaction conditions gave **14R** mainly as the β-anomer. The fact that an inversion of configuration of the asymmetric center of the auxiliary led to a reversal of the stereochemical outcome of the glycosylation provided strong support for the proposed mode of participation. In order to demonstrate the generality of the approach, a range of glycosyl acceptors was glycosylated with **5R** and **5S**. In each case, a glycosylation with glycosyl donor **5S** gave mainly an α-anomer whereas the use of **5R** led to the formation of β-anomers.

It is well known that the protecting group pattern of a glycosyl donor can influence the anomeric outcome of a glycosylation. Therefore, the glucosyl donors **6R/S**, **7R/S** and **8R/S** were prepared which have a benzoyl ester, an allyloxycarbonate or an allyl ether at C-3, respectively. These glycosyl donors

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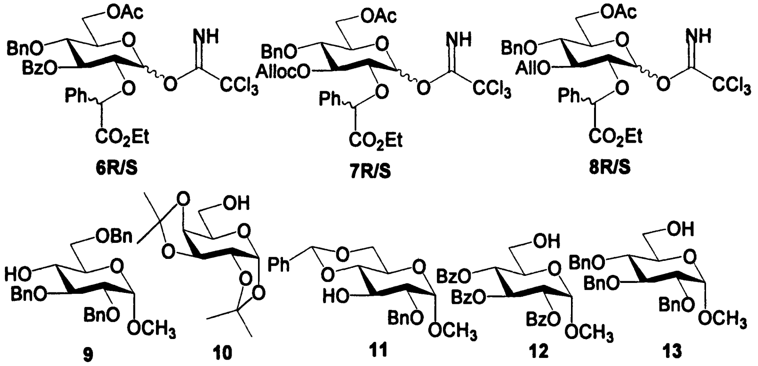
were expected to be convenient for complex oligosaccharide synthesis because the C-6 acetyl ester can be removed in the presence of a benzoyl group whereas the allyl- or allyloxycarbonyl protecting group can be cleaved without affecting the acetyl group at C-6. These glycosyl donors could easily be prepared from the key intermediates **2R/S**.

Table 1. Stereoselective glycosylations with glucosyl donors 5R/S, 6R/S, 7R/S and 8 R/S



6R/S **7R/S** **8R/S**

9 **10** **11** **12** **13**

Entry	Glycosyl Donor	Glycosyl Acceptor	Product	α/β Ratio (% yield)[a]
1	5S	9	14S	20/1 (89%)
2	5R		14R	1/5 (91%)
3	5S	10	15S	12/1 (92%)
4	5R		15R	1/3 (88%)
5	5S	11	16S	10/1 (95%)
6	5R		16R	1/8 (94%)
7	5S	12	17S	18/1 (94%)
8	5R		17R	1/1 (89%)
9	5S	13	18S	4/1 (93%)
10	5R		18R	1/6 (96%)
11	6S	9	19S	7/1 (88%)
12	6R		19R	1/3 (94%)
13	6S	10	20S	α (93%)
14	6R		20R	β (84%)
15	7S	9	21S	6/1 (81%)
16	7R		21R	1/9 (85%)
17	7S	10	22S	10/1 (79%)
18	7R		22R	β (90%)
19	8S	9	23S	1/3 (87%)
20	8R		23R	1/4 (80%)
21	8S	10	24S	4/1 (95%)
22	8R		24R	1/5 (75%)

[a] Product ratios were determined by ^{1}H NMR analysis of the crude reaction products.

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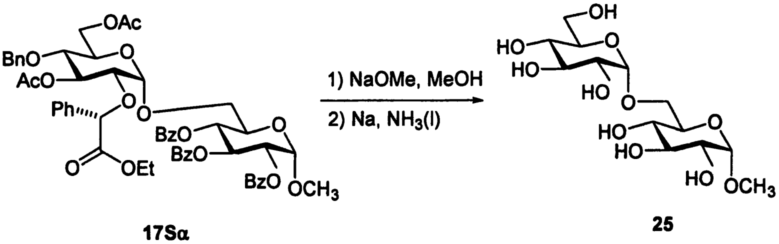
As can be seen in Table 1, the glycosylations with glycosyl donors **6S** and **7S** gave the corresponding disaccharides **19S, 20S, 21S** and **22S** in excellent yields with good to exclusive α-anomeric selectivity (Table 1, entries 11, 13, 15 and 17). When the corresponding **6R** and **7R** were employed as glycosyl donors an expected reversal of anomeric selectivity was observed and the disaccharides **19R, 20R, 21R** and **22R** were isolated as mainly β-anomers (Table 1, entries 12, 14, 16 and 18). Surprisingly, the glycosylations of **8S/R**, which have an allyl ether at C-3, with glycosyl acceptors **9** and **10** gave the corresponding disaccharides **23S/R** and **24S/R** with modest anomeric selectivity (Table 1, entries 19-22). These results indicate that an ester-protecting group at C-3 is important for efficient participation of the (*S*)-ethoxycarbonylbenzyl auxiliary.

The auxiliary was designed in such a way that it contains a substituted benzyl ether, and therefore should be removable by catalytic hydrogenation over Pd/C or Birch reduction. Indeed, saponification of the benzoyl ester of **17S(α)** by treatment with NaOMe in methanol followed by removal of the benzyl ethers and chiral auxiliary using sodium in liquid ammonia led to a clean formation of the deprotected compound **25** (Scheme 4). On the other hand, catalytic hydrogenation over Pd/C was sluggish probably due to steric hindrance at the benzylic carbon.



Scheme 4. Deprotection of disaccharide 17Sα

The novel methodology could also be applied to galactosyl donors. As expected, a TMSOTf mediated glycosylation of **26S** with glycosyl acceptors **9** and **12** gave the corresponding disaccharides **27S** and **28S** with good α-anomeric selectivity whereas the use of the glycosyl donor **26R**, which has a *R*-mandelate at C-2, gave **27R** and **28R** with modest β-selectivity (Table 2).

The anomeric selectivities summarized in Table 1 and Table 2 show that glycosylations with glycosyl donors having an (*S*)-ethoxycarbonylbenzyl moiety at C-2 give predominantly α-glycosides whereas the donors containing an auxiliary with opposite stereochemistry give mainly β-glycosides. It was,

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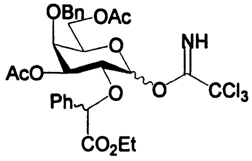
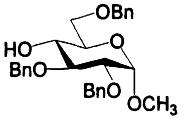
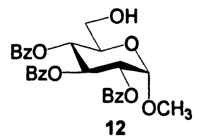
Table 2. Stereoselective glycosylations with galatosyl donors 26R/S



26R/S



9



12

Entry	Glycosyl Donor	Glycosyl Acceptor	Product	α/β Ratio (% yield)[a]
1	26S	9	27S	10/1 (75%)
2	26R		27R	1/4 (79%)
3	26S	12	28S	6/1 (78%)
4	26R		28R	1/2 (82%)

[a] Product ratios were determined by ^{1}H NMR analysis of the crude reaction products.

however, observed that the β-anomeric selectivities were somewhat lower. These glycosylations are proposed to proceed through a *cis*-decalin intermediate, which places the phenyl substituent of the auxiliary in an equatorial orientation (Scheme 2c). It is, however, important to note that a *cis*-decalin does experience unfavorable gauche interactions. The (*S*)-ethoxycarbonylbenzyl auxiliary, which reacts through a *trans*-decalin intermediate, does not experience these unfavorable interactions rationalizing its more efficient anomeric control.

In general, participation by conventional C-2 ester leads to the exclusive formation of 1,2-*trans* glycosides. Although the glycosyl donors that have an ethyl *S*-mandelate at C-2 led to disaccharides with high α-anomeric selectivity, small amounts of the unwanted β-anomers were formed. The formation of the unwanted anomer was significant when glycosyl donor **8S** was used, which has an allyl ether at C-3. Participation by an ethoxycarbonylbenzyl moiety is probably slower than that of a conventional ester because of slower six-membered than five-membered ring formation. Thus, it is possible that in the case of an ethoxycarbonylbenzyl participating group, some glycosylation takes place from the oxacarbenium ion leading to anomeric mixtures. This proposal is supported by the observation that dilution of the reaction mixture resulted in an improvement of anomeric selectivity. The oxacarbenium ion is disfavored by electron withdrawing substituent such as an ester at C-3, which may facilitate the participation. Differences in stability of the intermediate oxacarbenium ion may rationalize the more efficient participation of glycosyl donors **5S**, **6S** and **7S** compared to **8S**.

Although glycosylations with **5S** led to disaccharides with high α-anomeric selectivity, the ultimate goal is the development of an auxiliary that gives only one of the two possible anomers. In order to achieve this goal, we have examined a number of alternative auxiliaries. We have obtained the most promising results

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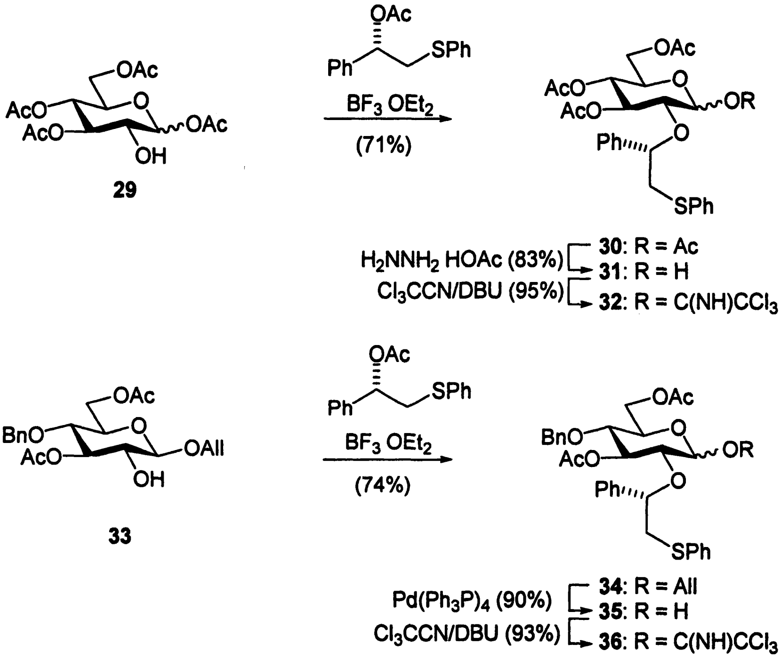
with a (*S*)-(phenylthiomethyl)benzyl moeity.[42] This auxiliary could easily be installed by reaction of a sugar alcohol, such as **29**[43] and **33**[44], with (*S*)-(phenylthiomethyl)benzyl acetate in the presence of BF_3-OEt_2.[45] This reaction proceeds by a BF_3-OEt_2 promoted departure of the acetyl ester with concomitant formation of an episulfonium ion. Subsequently, nucleophilic attack at the benzylic position of the episulfonium ion by a sugar hydroxyl leads to the required substituted benzyl ether with overall retention of configuration. Detailed NMR analysis of products **30** and **34** revealed that no other regio- or stereoisomers had been formed. Compounds **30** and **34** could be converted into glycosyl donors **32** and **36** by either removal of the anomeric acetyl ester or allyl ether followed by conversion of the hemiacetals into anomeric trichloroacetimidate using standard reaction conditions (Scheme 5).[46]

Coupling of **32** or **36** with glycosyl acceptor **12** using catalytic amount of TMSOTf in dichloromethane at –78°C followed by gradual warming to 10°C gave, after a reaction time of 3 hrs, disaccharides **37** and **38** as only the α-glycosides in good yields. The generality of the approach was demonstrated by the coupling of a range of glycosyl acceptors with **32** and **36** and, in each case only the expected α-anomer was isolated (Scheme 6).



OAc
AcO
AcO
OAc
OH
29
OAc
Ph
SPh
BF3 OEt2
(71%)
OAc
AcO
AcO
OR
Ph
O
SPh
H2NNH2 HOAc (83%)
Cl3CCN/DBU (95%)
30: R = Ac
31: R = H
32: R = C(NH)CCl3
OAc
BnO
AcO
OAll
OH
33
OAc
Ph
SPh
BF3 OEt2
(74%)
OAc
BnO
AcO
OR
Ph
O
SPh
Pd(Ph3P)4 (90%)
Cl3CCN/DBU (93%)
34: R = All
35: R = H
36: R = C(NH)CCl3

Scheme 5. Preparation of glycosyl donors 32 and 36

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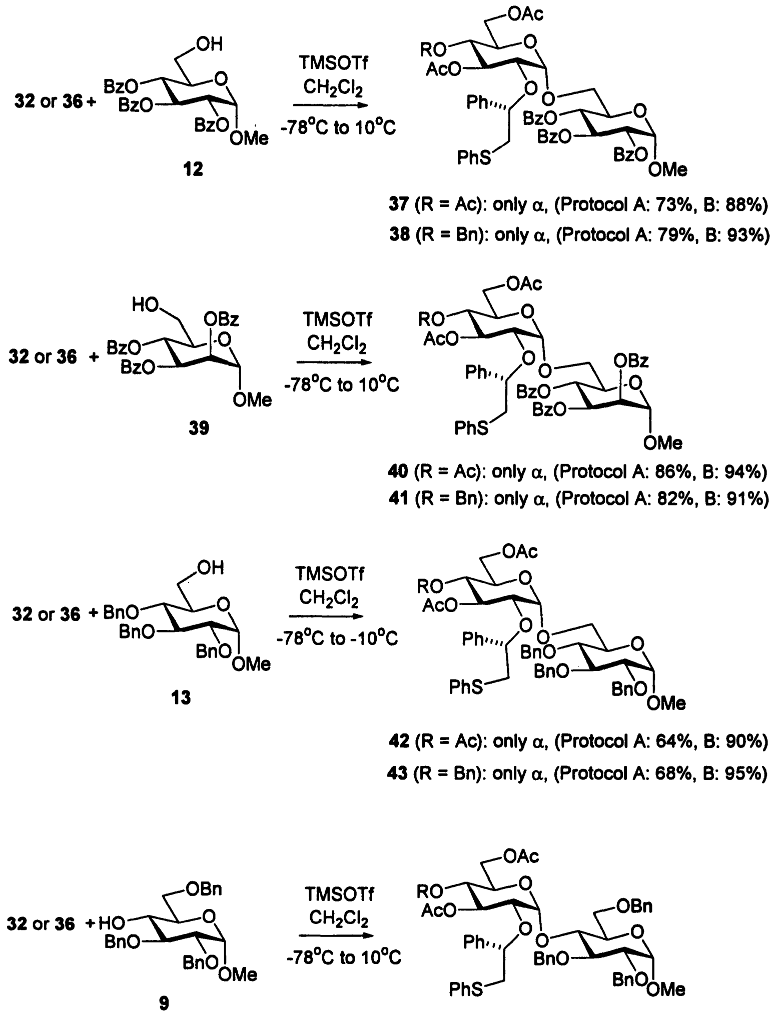

12

37 (R = Ac): only α, (Protocol A: 73%, B: 88%)
38 (R = Bn): only α, (Protocol A: 79%, B: 93%)

39

40 (R = Ac): only α, (Protocol A: 86%, B: 94%)
41 (R = Bn): only α, (Protocol A: 82%, B: 91%)

13

42 (R = Ac): only α, (Protocol A: 64%, B: 90%)
43 (R = Bn): only α, (Protocol A: 68%, B: 95%)

9

44 (R = Ac): only α, (Protocol A: 62%, B: 83%)
45 (R = Bn): only α, (Protocol A: 70%, B: 85%)

Scheme 6. Stereoselective glycosylations with glycosyl donors 32 and 36

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It was observed that some degradation occurred during the glycosylations probably due to the acidic nature of the reaction conditions. This problem could be addressed by an alternative glycosylation protocol (Scheme 6, protocol B) whereby the glycosyl donor was first activated with TMSOTf followed by the addition of the acceptor in the presence of the base 2,6-di-*tert*-butyl-4-methyl pyridine. Under these conditions no degradation was observed and in each case, the disaccharides were isolated in improved and near quantitative yields.

The formation of an intermediate equatorially substituted anomeric sulfonium ion (**46**) was demonstrated by treatment of glycosyl donor **36** in CD_2Cl_2 with one equivalent of TMSOTf followed by recording ^{1}H, ^{1}H-TOCSY, HSQC and HMBC NMR spectra. The combined NMR data showed the formation of a single new compound, which was unambiguously identified as the β-substituted sulfonium ion. Treatment of the sulfonium ion with methanol resulted in the clean formation of the corresponding methyl α-glycoside, demonstrating that the glycosylation proceeds by inversion of configuration of the anomeric center (Scheme 7).

A number of experiments established which features of the (*S*)-(phenylthiomethyl)benzyl moiety are important for controlling the α-anomeric selectivity. In this respect, a reaction of an intermediate oxacarbenium ion with an externally delivered sulfide may also lead to the formation of an equatorially substituted sulfonium ion, which may be displaced by a sugar alcohol to give an α-glycoside. Furthermore, the chiral center of the (*S*)-(phenylthiomethyl)benzyl moiety may not be essential for achieving absolute α-anomeric selectivity and it may well be possible that *trans*- *vs.* *cis*-decalin formation in combination with stereo-electronic effects is sufficient to induce the formation of a β-substituted sulfonium ion reacting to an α-glycoside. To investigate these issues, trichloroacetimidate donors **48, 50** and **52** were coupled with glycosyl acceptor **13** using TMSOTf as a promoter (Scheme 8). A standard glycosylation of trichloroacetimidate **48**, which has a benzyl ether at C-2, gave the disaccharide **49** as a 3/1 mixture of α/β anomers. When the glycosylation was performed in the presence of ethyl phenyl sulfide (5 equivalents), only a marginal increase in α-anomeric selectivity (α/β = 6/1) was observed. Next, a glycosylation was performed with glycosyl donor **50**, which has an unsubstituted phenylthioethyl ether at C-2. In this case, the disaccharide **51** was obtained as a 8/1 mixture of α/β anomers. A similar glycosylation with **52**, having a (*R*)-(phenylthiomethyl)benzyl at C-2, resulted in the formation of **53** as an anomeric mixture (α/β = 1/1). Thus, unlike the (*R/S*)-ethoxycarbonylbenzyl auxiliary, the use of a C-2 (phenylthiomethyl)benzyl seems to be limited to the synthesis of 1,2-*cis* glycosides. This is not a problem because the alternative *trans*-glycosides can be obtained by classical neighboring group participation using ester-protecting groups.

Collectively, these results support the proposed mode of anomeric control by a (*S*)-(phenylthiomethyl)benzyl moiety at C-2 of a glycosyl donor. The new glycosylation protocol described here in combination with traditional

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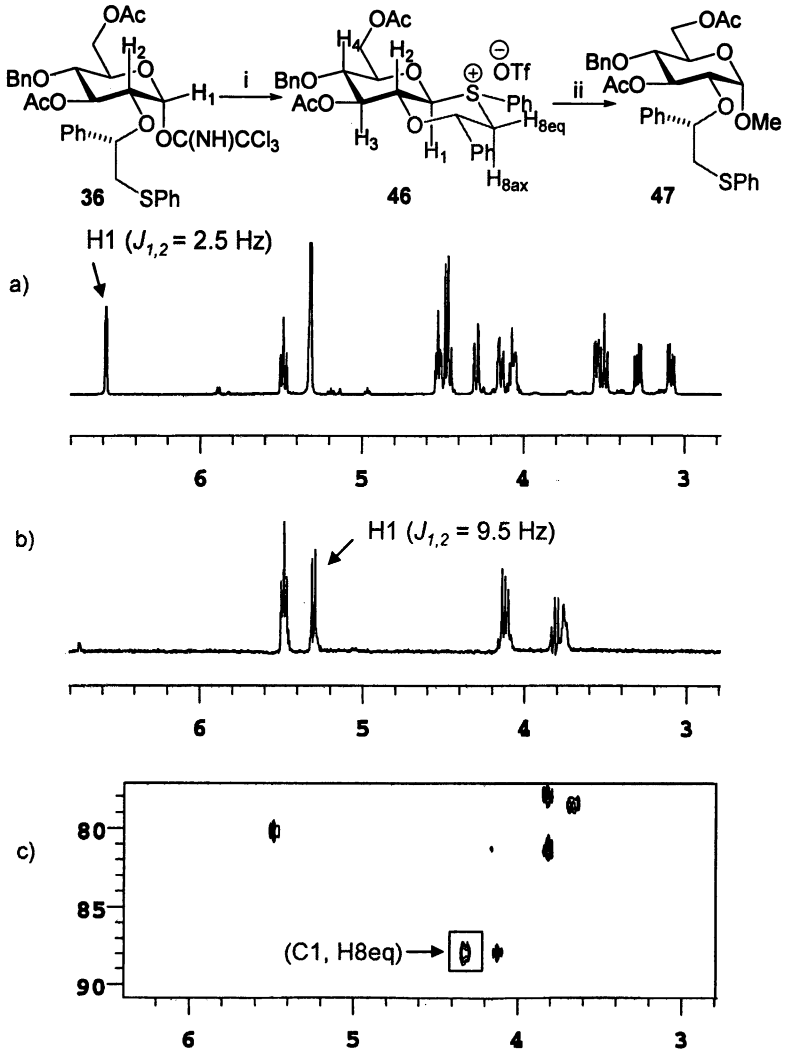
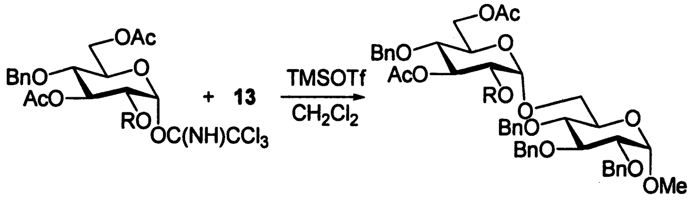


Scheme 7. i) TMSOTf, CD$_2$Cl$_2$, -50°C to 0°C ii) MeOH, -20°C to 0°C a) ^{1}H NMR spectrum of glycosyl donor 36 b) ^{1}H TOCSY 1D spectrum on irradiation of H4 of sulfonium ion 46 c) HMBC spectrum of sulfonium ion 46

neighboring group participation by esters should allow the installment of α- as well as β-glycosides. The combined use of these methodologies to control anomeric selectivities was demonstrated by the synthesis of trisaccharide **57**, which has been identified as an epitope that can trigger acute rejections in xenotransplantation (Scheme 9).[47,48] This compound was successfully assembled from the monomeric building blocks **54**, **55** and **56** using a one-pot two-step glycosylation sequence as shown in Scheme 9. Interestingly, the NIS/TMSOTf promoter system did not affect the (*S*)-(phenylthiomethyl)benzyl group.



48: R = Bn	**49**: R = Bn
	a. TMSOTf, CH_2Cl_2, α/β = 3/1 (94%)
	b. TMSOTf, CH_2Cl_2, PhSEt, α/β = 6/1 (92%)
50: R = $(CH_2)_2SPh$	**51**: R = $(CH_2)_2SPh$, α/β = 8/1 (86%)
52: R = (*R*)-CH(Ph)CH_2SPh	**53**: R = (*R*)-CH(Ph)CH_2SPh, α/β = 1/1 (88%)
36: R = (*S*)-CH(Ph)CH_2SPh	**43**: R = (*S*)-CH(Ph)CH_2SPh, α only (95%)

*Scheme 8. Glycosylations with glycosyl donors **48**, **50**, **52** and **36***

Finally, it was showed that the (*S*)-(phenylthiomethyl)benzyl group could be removed by treatment with BF_3-OEt_2 in acetic anhydride to give the corresponding acetate. The deprotection led to a full recovery of (*S*)-(phenylthiomethyl)benzyl acetate, which could be reused for the installment of an (*S*)-(phenylthiomethyl)benzyl moiety.

It has been shown that the use of chiral auxiliaries such as a (*S*)-(phenylthiomethyl)benzyl moiety at C-2 of a glycosyl donor can control the formation of α-glycosides. A combined use of the new approach to introduce α-glycosides and traditional neighboring group participation by C-2 esters to give β-glycosides provides a strategy for the synthesis of a wide variety of oligosaccharides. It is also important to note that reliable approaches for the stereoselective glycosylation of sialic acids and β-mannosides have been reported. It is to be expected that stereoselective glycosylation protocols for complex oligosaccharide synthesis will facilitate progress in glycobiology.

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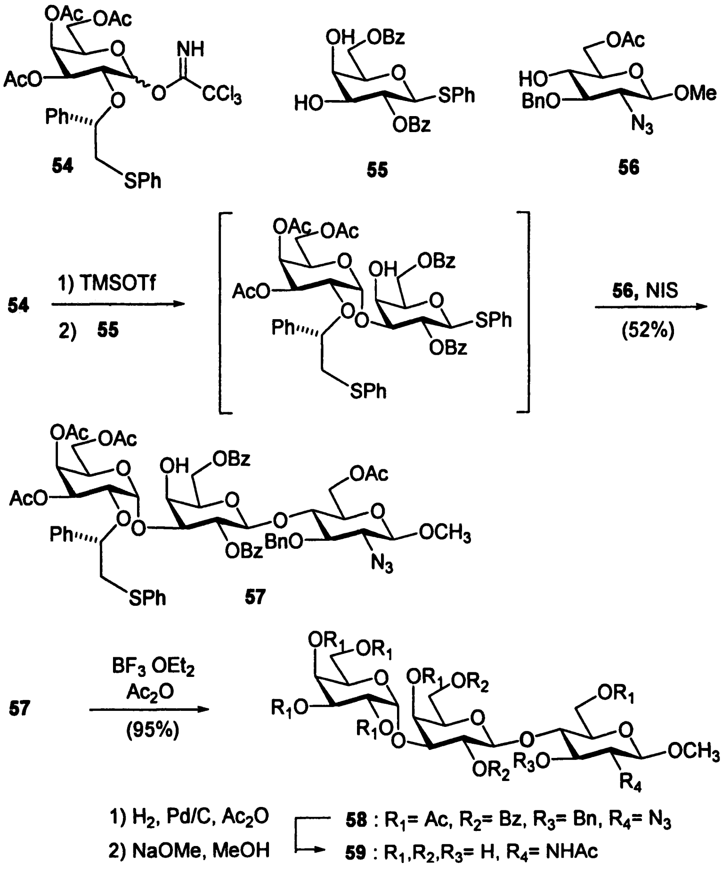
54
55
56
54
1) TMSOTf
2) 55
56, NIS
(52%)
57
57
BF_3 OEt_2
Ac_2O
(95%)
1) H_2, Pd/C, Ac_2O
2) NaOMe, MeOH
58 : R_1= Ac, R_2= Bz, R_3= Bn, R_4= N_3
59 : R_1,R_2,R_3= H, R_4= NHAc

Scheme 9. One-pot two-step synthesis of trisaccharide 57 using glycosyl donor 54 and deprotection

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Chapter 7

Protecting Groups in Carbohydrate Chemistry Profoundly Influence All Selectivities in Glycosyl Couplings

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Three selectivities, stereo, chemo and regio, that confront organic synthesis in general, are encountered in glycosidation reactions. In these reactions, protecting groups are a necessary evil; but they do more than protect, for they profoundly affect all three selectivities. Case studies show that the "normal" primary *versus* secondary hydroxyl preference holds only for disarmed donors, whereas armed donors are frequently secondary-OH selective. These selectivities are so reliable, that a three-component, in situ competition leads to a single double glycosidation product in which each donor goes to its preferred-OH.

[¥] NPG is an independent, non-profit research facility with laboratories at Centennial Campus (North Carolina State University), Raleigh, NC USA.

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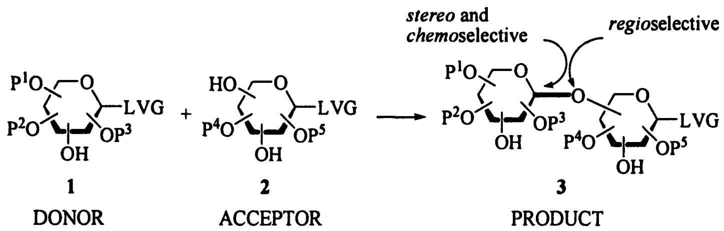
ACS Symposium Series; American Chemical Society: Washington, DC, 2007.

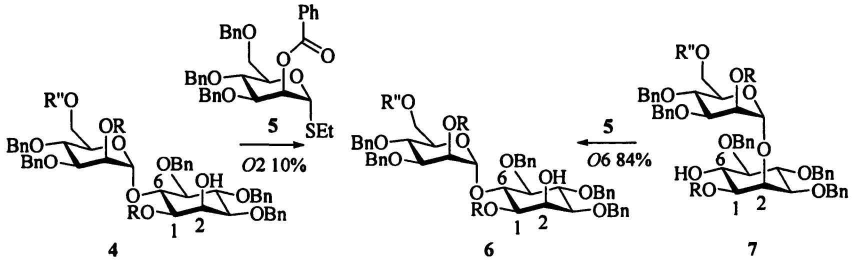
Introduction

The hypothetical glycosidation shown in Scheme 1 entails three of the four modes of selectivity, stereo, chemo and regio that, according to Trost (*1*), confront organic synthesis in general. The fourth, enantioselectivity, is usually not encountered in oligosaccharide synthesis since the chiralities of the donor and acceptor are usually specified by nature.



Scheme 1. General glycosidation protocol.

The problem of stereoselectivity received early insight from Isbell's monumental 1941 paper "*Sugar Acetates, Acetylglycosyl Halides and Orthoacetates in Relation to the Walden Inversion*" (*2b*). This landmark publication, and its 1940 companion publication *(2a),* identified the role of a donor's 2-*O*-acyl protecting group on the configurational outcome at the anomeric center in Koenigs-Knorr reactions of acetylated glycosyl halides (see Scheme 3).



Scheme 2. An example from van Boom and coworkers.

Insight into *chemo* and *regio*selectivities in donor/acceptor coupling has been less forthcoming as the examples from van Boom's work in Scheme 2 illustrate (*3,4*). Thus, the 84% versus 10% yields in the reaction of the same donor, 5, with acceptors 4 and 7 do not succumb to facile "more versus less

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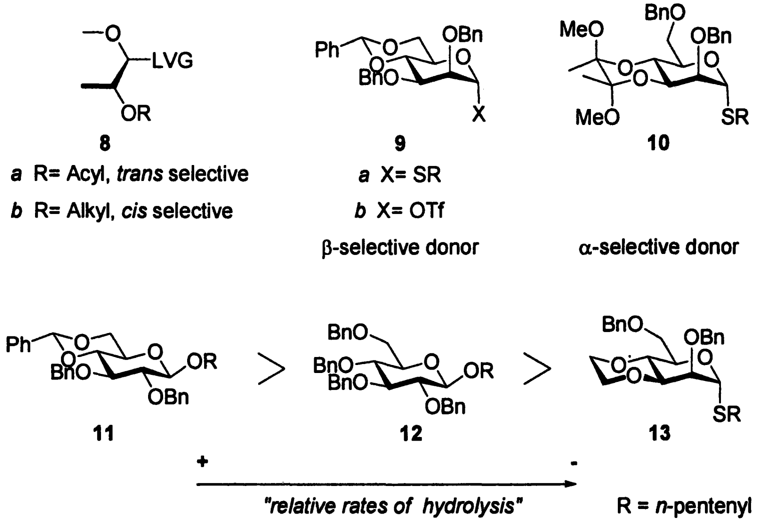
reactive," or "steric hindrance" rationalizations. Indeed, the example in Scheme 2 justifies Paulsen's 1982 advice that "each oligosaccharide synthesis --- requires considerable systematic research and a good deal of know how," (*5*) and his subsequent notion that donor and acceptor must "match" for best results (*6*). Paulsen could not specify the requirements for such a match, given the small data base available at the time; but they could certainly be recognized after the event. For example, donor **5** matches *O*6-OH of **7**, but not *O*2-OH of **4**.

Attempts are underway in our laboratories to gain insight into the requirements for donor/acceptor match, and some pertinent observations are discussed in this article.

Protecting Groups do More than Protect

The aforementioned 1941 paper of Isbell (*2b*) was a landmark on several fronts. The mechanistic postulate contributed massively to what has come to be known as neighboring group participation (*7*). No less seminal, although not immediately appreciated, was the inherent message that with carbohydrate derivatives "protecting groups do more than protect". Indeed, the only implements for influencing the various selectivities in Scheme 1 are the protecting groups of the reactants.



LVG
OR
8
a R= Acyl, trans selective
b R= Alkyl, cis selective
Ph
OBn
BnO
X
9
a X= SR
b X= OTf
β-selective donor
MeO
BnO
OBn
MeO
SR
10
α-selective donor
Ph
OR
BnO
OBn
11
>
BnO
BnO
BnO
OR
OBn
12
>
BnO
OBn
SR
13
+
-
"relative rates of hydrolysis"
R = n-pentenyl

Scheme 3. Protecting Groups in Glycosyl Donors.

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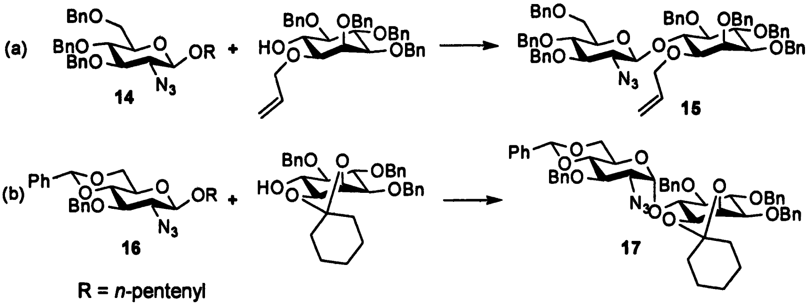
The Donor

Isbell's observation evolved into the "rule of thumb" that *O2* participating and non-participating groups promote *trans* and *cis* couplings, respectively, as indicated for **8a** and **b** in Scheme 3. Famously, β-mannosides remained spectacular exceptions to the rule. However, the solution of that stubborn problem was again found to lie in the choice of protecting group of the mannosyl donor. Thus, the elegant studies of Crich on the mechanism of his β-mannoside synthesis, showed that a 4,6-*O*-benzylidene group, as in **9**, was essential (*8*). Interestingly, when the "protecting group" was shifted to the *O3*/*O4* position as in **10**, only α-linked products were obtained (*9*).

As noted by Crich, the observed α versus β stereoselectivities observed for **9** and **10**, comport with the relative rates of oxidative hydrolysis reported by Fraser-Reid and coworkers (*10*) for the *n*-pentenyl glycosides **11**, **12** and **13**. Thus, the torsional strain imposed by the 4,6-*O*-benzylidene "protecting group" allows the critical α-triflate intermediate **9b**, formed *in situ,* to exist long enough for "Sn^2-like displacement" (*8*) to occur leading to the β-mannoside.



Reagents and conditions: NIS, TESOTf, (a) 1 h, <10%, (b) 10 days, 59%

Scheme 4. Torsional effects of the glycosyl donor on stereoselectivity.

If formation and rate of reaction of the α-triflate **9b** is indeed the determining factor, could torsional factors be utilized to optimize α-stereoselective coupling?

The results in Scheme 4 suggest the affirmative (*11*). Thus, the conformationally mobile donor **14** gave mainly β-product **15** in poor yield; however, the torsionally disarmed counterpart **16** gave a two-fold higher yield of α-product **17**(*12*). The slow rate of reaction attributable to the torsional strain suffered by **16**, is consistent with the long- lived intermediate **9b** in Crich's β-mannoside synthesis.

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The Acceptor

For the acceptor **2**, the other partner in the reaction in Scheme 1, all hydroxyl groups, except the targeted one(s) are usually protected. Benzyl is the traditional "permanent" protecting group, for it must survive until the final step where it is removed to provide the "free glycan".

However, since the acceptor-OH is a nucleophile, the choice of protecting groups, P^4 and P^5 in diol **2**, could affect the electron density of the adjacent–OH. Indeed, our own experiments shown in Scheme 5, indicate how the change of "protecting group" of the acceptor can influence yields (*13*). Thus while the acetate in **19a** gave only 37% yield of **20a**, use of the benzylated analogue **19b** more than doubled the yield of **20b**.

Unfortunately, the protecting group advantage of **19b** over **19a** cannot usually be exploited, because the logistics of tampering with protecting groups midway through the synthesis of an oligosaccharide would present bewildering problems.

Attempts to correlate acceptor–OH activity with infrared data have been made (*14*); but we are not aware of any major synthesis that has been based on these data.

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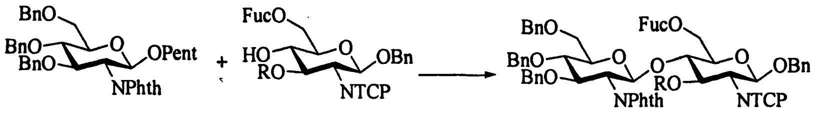

18

19
a R = Ac
b R = Bn

20
a R = Ac (37%)
b R = Bn (77%)

Reagents and conditions : NIS, TESOTf, -20° C

Scheme 5. Influence of the protecting groups on the glycosyl acceptor.

Chemoselectivity

The Armed/Disarmed Enigma

The experiment in Scheme 6a was undertaken by van Boom and co-workers (*15*) to show that the armed/disarmed protocol, discovered with *n*-pentenyl glycosides (Scheme 6b) (*16*), could be extended to thioglycosides.

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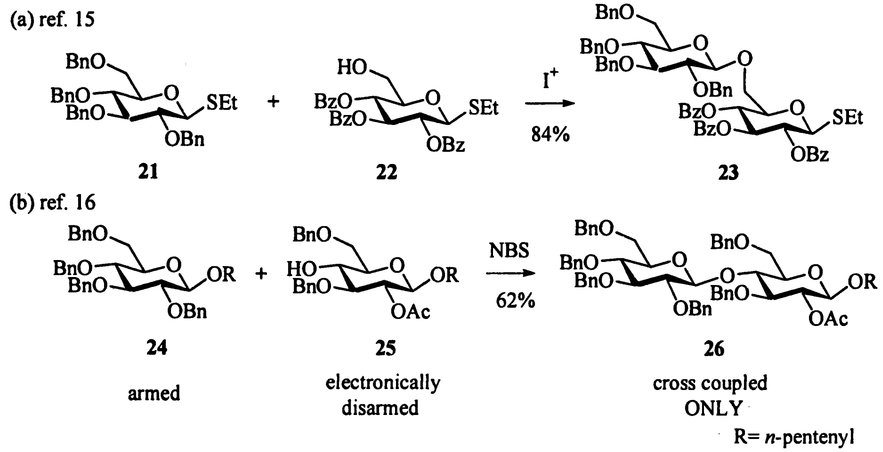

(a) ref. 15
BnO
BnO
BnO
O
SEt
OBn
21
+
HO
BzO
BzO
O
SEt
OBz
22
I+
84%
BnO
BnO
BnO
O
O
OBn
BzO
BzO
O
SEt
OBz
23
(b) ref. 16
BnO
BnO
BnO
O
OR
OBn
24
armed
+
BnO
HO
BnO
O
OR
OAc
25
electronically disarmed
NBS
62%
BnO
BnO
BnO
O
OBn
BnO
O
BnO
O
OR
OAc
26
cross coupled ONLY
R= n-pentenyl

Scheme 6. Armed-Disarmed glycosyl couplings.

The First Rationalization

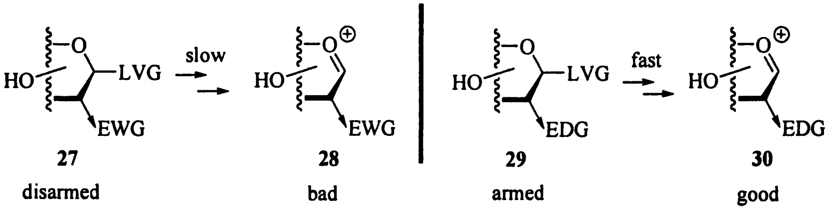
Conflict with Anchimeric Assistance

The original rationalization (*17*) of the armed/disarmed chemoselectivity (Scheme 7a) was attributed to the electron withdrawing (disarming) effect of the *O*2 substituent of **27** which curtailed progress to the oxocarbenium ion **28**, whereas the *O*2-alkylated counterpart **29** proceeded rapidly, leading to a higher concentration of **30**. Accordingly, in a competitive situation, cross-coupling, **27+30**, would be more likely to occur than self-coupling, **27+28**.

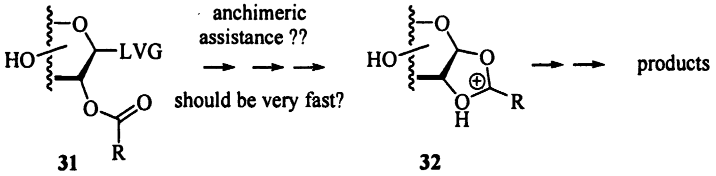
There was, however, a niggling problem with such a rationalization, which is outlined in Scheme 7b. The stereochemical aspect of neighboring group participation, is (sometimes) twinned with the kinetic aspect termed anchimeric assistance (*18*). Thus, the reaction of **31** should proceed through a dioxolenium intermediate **32** en route to the *trans* product. This assistance should be optimal for a *trans*-1,2 donors such as **22** and **25**, and should cause them to react *faster* than the armed counterparts **21** and **24**. Self-coupling of **22** and **25** should therefore have been the principal reaction pathways!

In an attempt to clarify the conflict between Scheme 7a and 7b, theoretical studies were carried out using the tetrahydropyran derivatives in Figure 1 as models (*19*). The ethoxy chlorides, **33** and **36**, correspond to armed donors, and acetoxy counterparts, **34** and **37**, to the disarmed analogs. The corresponding oxocarbenium ions are **39**/**43**, and **40**/**44** respectively.

(a) First rationalization (1990)


HO
O
LVG
EWG
slow
HO
O⊕
EWG
HO
O
LVG
EDG
fast
HO
O⊕
EDG
27
disarmed
28
bad
29
armed
30
good

(b) However when EWG = Acyl


HO
O
LVG
O
O
R
anchimeric
assistance ??
should be very fast?
HO
O
O
⊕
O
H
R
products
31
32

Scheme 7. Armed-Disarmed effects and anchimeric assistance.

The transition energies, shown along the broken arrows in Figure 1, affirm that the armed donors should ionize more readily than their disarmed counterparts. However if **34** and **37** should avail themselves of anchimeric assistance, they would proceed to **41**/**42** and **45** with an energetic advantage of 8-9 kcal over their armed competitors **33** and **36**.

By corollary, the formation of **23** and **26** in Scheme 6 means that the anchimeric assistance pathways via dioxolenium ions (Figure 1, and Scheme 7b) were not followed.

An Alternative Rationalization

(Cyclic) Iodonium Ion Transfer

In view of the dilemma apparent in Schemes 7a and b, an alternative rationalization was required, and this emanated from our studies on the oxidative hydrolysis of ω-alkenyl glycosides, **46a-d** (Scheme 8) (*20*). We found that all of these alkenes underwent addition reactions to give bromohydrins, **47**, with the spectacular exception of pentenyl, **46c**, which underwent hydrolysis to give the corresponding glycose **48**.

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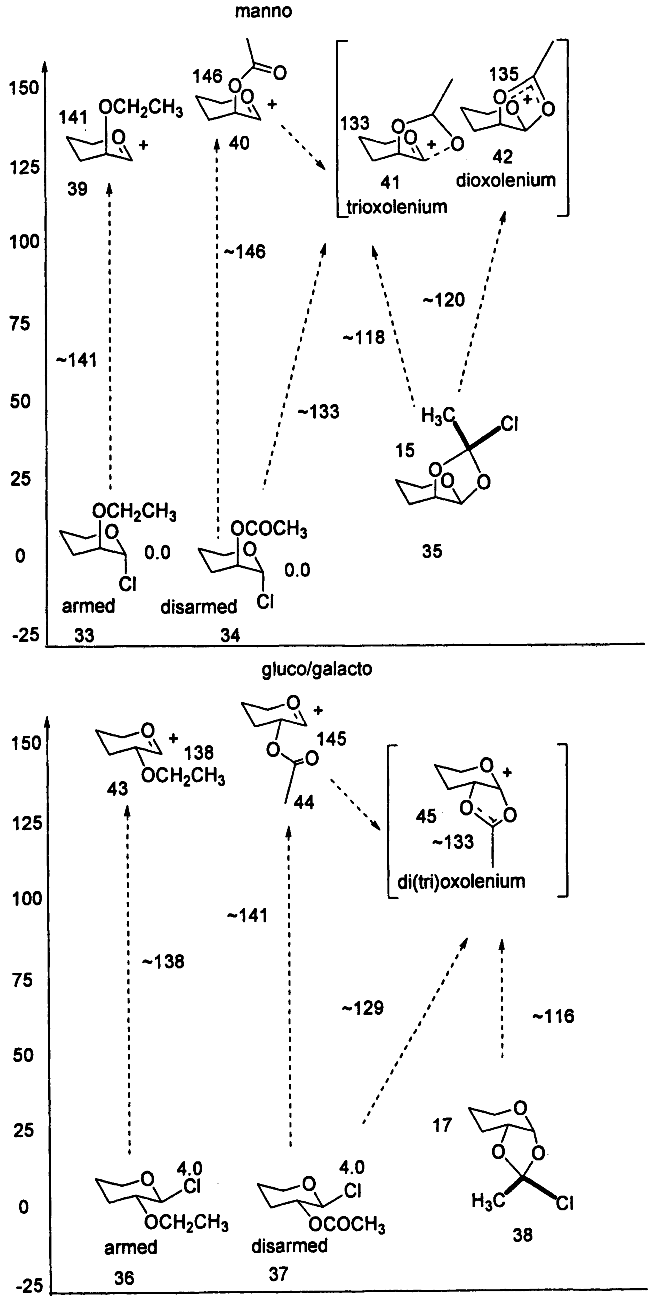
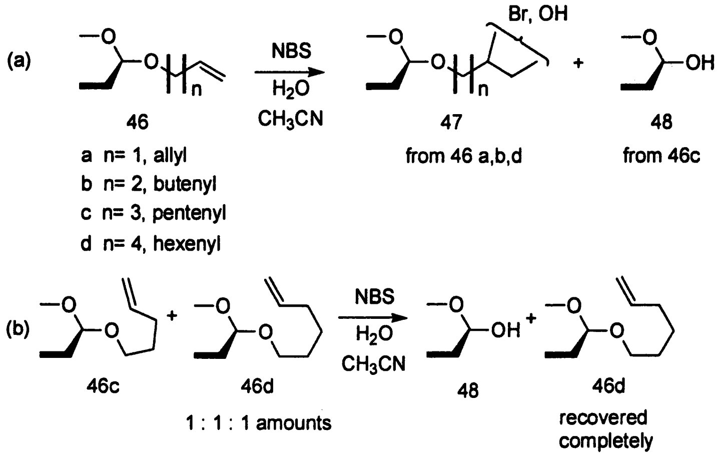

manno
150
125
100
75
50
25
0
-25
141
OCH2CH3
O
+
39
146
O
O
+
40
~141
~146
133
O
+
O
41
trioxolenium
135
O
+
O
42
dioxolenium
~133
~118
~120
H3C
Cl
15
O
O
O
35
OCH2CH3
O
0.0
Cl
armed
33
OCOCH3
O
0.0
Cl
disarmed
34
gluco/galacto
150
125
100
75
50
25
0
-25
O
+
138
OCH2CH3
43
O
+
145
O
O
44
O
+
45
O
O
~133
di(tri)oxolenium
~138
~141
~129
~116
O
4.0
Cl
OCH2CH3
armed
36
O
4.0
Cl
OCOCH3
disarmed
37
17
O
O
O
H3C
Cl
38

Figure 1. Armed-Disarmed effects and anchimeric assistance.



Scheme 8 (ref. 20). ω-Alkenyl glycosides.

In the course of these studies, we found that when **46c** and **46d** were made to compete for one equivalent of NBS (Scheme 8b), the former was completely hydrolyzed, while the latter was completely recovered. The efficiency of this transfer was remarkable, since the rate ratio for reaction of **46c** and **46d** separately with NBS was only ~2:1 (*20*).

Intermolecular transfer of bromonium ion, shown in Figure 2 had been observed by Brown for hindered alkenes (*21*); but the experiment in Scheme 8b showed that even terminal alkenes could experience this phenomenon.

Clearly therefore, halonium ion transfer could be operating between armed/disarmed NPGs **24** and **25** (Scheme 6b). As summarized in Figure 2, we can assume that portions of both armed and disarmed reactants gives rise to halonium ions, and that the incipient transition energies for the armed *versus* disarmed reactants, shown in Figure 1, are transmitted all the way back to these earliest intermediates. Thus the armed halonium ion progresses more quickly to products, thereby setting the stage for subsequent steady state halonium ion transfer.

The question of halonium ion transfer is pertinent to van Boom's experiment in Scheme 9a which pits thioglycoside **49** against NPG **50** (*22*). The high yield formation of **51** seemed obviously attributable to the fact that the sulfur of **49** is more readily oxidized by I^+ than the olefin of **50**. However, even this fundamental truth is subject to protecting group reversal, as the result in Scheme 9b shows (*23*). Thus, the electron-withdrawing benzoate groups completely negate the innate oxidative superiority of the sulfur in **53**, so that armed NPG **52** dominates in the competition for acceptor **54**, thereby producing **55a**.

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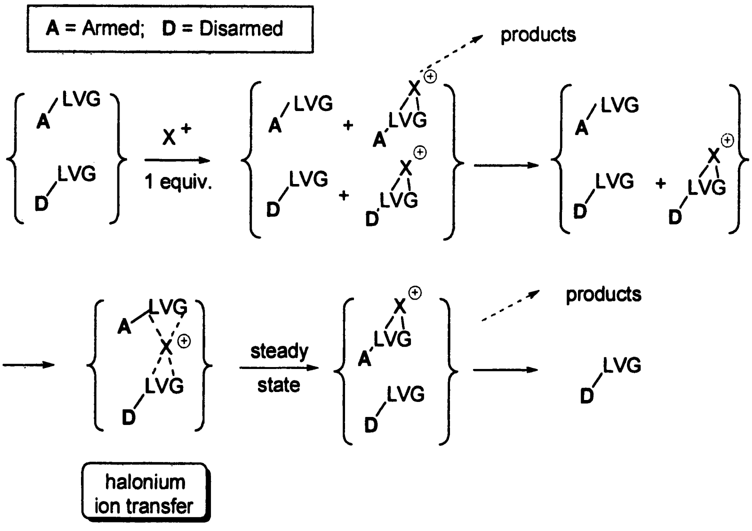

Figure 2. Intermolecular transfer of halonium ion permits complete reaction of one donor and recovery of the other.

The halonium ion transfer concept in Figure 2 is readily tested. The crucial steady state operation requires that only one equivalent of I^+ be used. A higher amount of NIS would disturb the distribution of intermediates, and this would disrupt the steady state transfer. This is evident in the tabulated data in Schemes 9b and c. Thus an increase to 3 equivalents of NIS, by eliminating the iodonium transfer between glycosyl donors, permits the (almost) simultaneous formation of oxocarbenium ions arising from **52** and **53**, with the result that thioglycoside **53** becomes the principal, donor with **55b** as major product (*23*).

What About Other Donors

Apart from *n*-pentenyl glycosides (*16*) and thioglycosides (*15*), disarmed couplings have been demonstrated for 2-pyridyl-1-thioglycosides, **56**, by Mereyala (*24*), and SBox glycosides, **57**, by Demchenko (*25*) (Scheme 10a).

However, we could find no report concerning the extensively used trichloroacetimidates (*26*). We therefore carried out the experiments with the trichloroacetimidates **58** and **59** in Scheme 10b. The disaccharide **60** most likely arose from the armed/disarmed product that suffered hydrolysis; but the low yield indicates that the armed/disarmed protocol operated much less well than the cases in Scheme 6.

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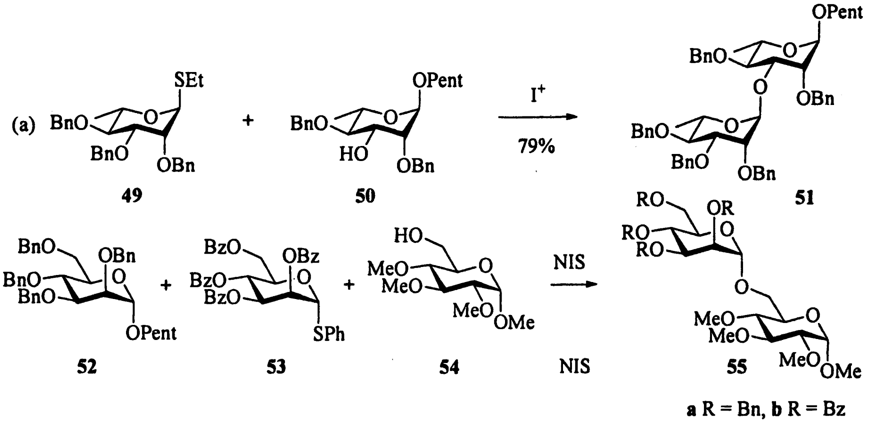


(b)	1 equiv	1 equiv	1 equiv	1 equiv	**55a** ONLY
(c)	1 equiv	1 equiv	1 equiv	3 equiv	**55a** (30%) **55b** (60%)

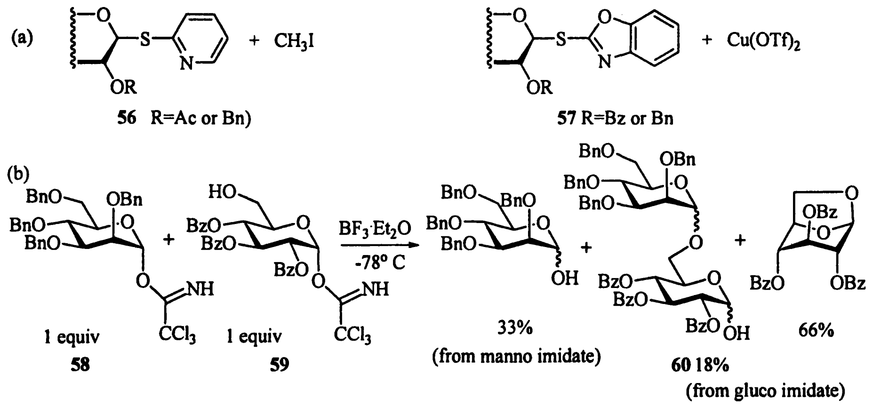
Scheme 9. Halonium ion transfer between n*-pentenyl glycosides and thioglycosides.*

In light of the above, there are (at least) two guidelines for predicting the success of armed/disarmed coupling. First, the energetics in Figure 1 imply that if the anchimeric assistance options open to **34** and **37** were to be taken, armed/disarmed coupling would **not** be observed. Given the variety of donors and/or reaction conditions that are available, it is reasonable to envisage situations where the armed/disarmed phenomenon would be inoperable.

Second, with respect to the rationalization based on Figure 2, the applicability to other electrophiles may be raised. From the experiments of Mereyala (*24*) and Demchenko (*25*) on their 2-pyridyl-thio (**56**) and SBox (**57**) glycosyl donors, it appears that $CH_3^+$and Cu^{++} are subject to transfer between competing substrates. On the other hand, although this remains to be tested, proton or Lewis acid transfer is unlikely to be steady-state determining, and hence could account for the disappointing results of armed/disarmed processes with the trichloroacetimidates in Scheme 10b.

"Moderately" Disarmed?

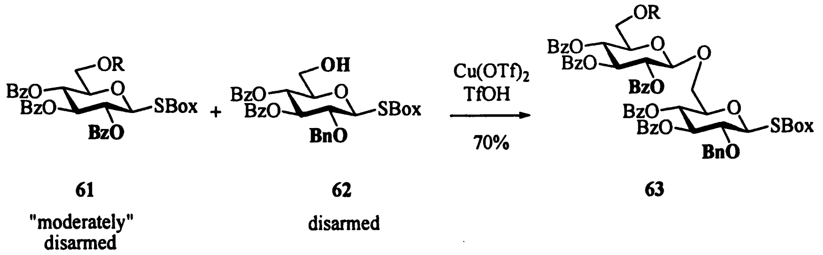
Since discovery of the armed/disarmed strategy for chemoselective coupling of sugars (*16*), several modifications have been tested to develop more nuanced effects (27). The process of discovery continues with a recent observation in Demchenko's lab shown in Scheme 11 (*28*). On the basis of the (now classical!) precedents shown in Scheme 6, the *O2*-benzoyl group of **61** and


(a)
+ CH3I
56 R=Ac or Bn)
+ Cu(OTf)2
57 R=Bz or Bn
(b)
+
BF3·Et2O
-78° C
1 equiv
58
1 equiv
59
+
+
33%
(from manno imidate)
60 18%
66%
(from gluco imidate)

Scheme 10. Armed-Disarmed processes with some other donors

O2-benzyl of **62** should be disarmed and armed respectively, and the outcome should have been self-coupling of **62**. This expectation was shattered by the formation of **63** in excellent yield.

Consequently Demchenko has described **61** as "moderately" disarmed, and **62** as disarmed. The prosecution of this concept invites further scrutiny.


+
Cu(OTf)2
TfOH
70%
61
"moderately"
disarmed
62
disarmed
63

Scheme 11. Demchenko's "moderately" disarmed donor.

Regioselectivity

We have reported several examples of regioselective glycosidation of diols (*29*). In order to facilitate comparisons, we maintained a "level playing field" by use of *n*-pentenyl donors. Furthermore, manno donors were studied first since,

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being α-selective, the analysis of the coupled products would be simplified. Our published studies (*29*) indicate that armed and disarmed donors display different regioselectivities, and that these differences are not based on the simple, traditional hydroxyl hierarchies, primary > secondary, equatorial > axial, or hindered > non-hindered. More recent studies, relevant to these hierarchies, will be presented below.

Lanthanide Triflates

But first, a major advance in our regioselectivity studies emanated from our interest in the use of lanthanide triflates as Lewis acid surrogates, in the hope that acid-labile protecting groups would not be affected. In this context, *n*-pentenyl orthoesters (NPOEs), *e.g.* **64**, were of special interest, since these donors are unique to the *n*-pentenyl family. NPOEs show the same regioselectivities as disarmed NPGs *e.g.* **65a** into which they are quantitatively rearranged (*29*); but NPOEs being infinitely more reactive, are worthy of special attention.

Of several salts studied, ytterbium and scandium triflates provided the greatest contrast for our purposes (*30*). Thus, both could be used to effect NPOE→disarmed NPG rearrangement **64**→**65a** (Scheme 12a), the latter being routinely converted into the armed counterpart **65b**.

When the NPG **65a** or **b** was treated with an alcohol, ROH, and NIS/Sc(OTf)$_3$ as promoter, the glycoside **66** (R=Bn or Bz) was formed, as expected. However when the promoter system was changed to NIS/Yb(OTf)$_3$, there was no reaction, indicating that armed and disarmed NPGs **65a** and **b** were refractory to Yb(OTf)$_3$.

The results in Scheme 12a suggests that a diol, **67**, could be treated with an NPOE, **64**, used in excess so as to optimize regioselective monoglycosidation leading to **68**. The excess NPOE would show up as the disarmed NPG **65a** which, being refractory to Yb(OTf)$_3$, would remain in solution as a spectator.

However if NIS/Sc(OTf)$_3$ were to be used, the double glycosidation product **69** would be formed since NPG **65a** would now be activated by the salt in solution.

The concept in Scheme 12b has been reduced to practice as exemplified in Scheme 13 (*31*) with diols **70**, **73** and **76**. The combination of NPOE, **64**, NIS and Yb(OTf)$_3$ was found to be exquisitely regioselective in all cases; but with NIS/Sc(OTf)$_3$ appreciable double glycosidation was observed. It is notable, that this trend held for competition between primary/secondary (Schemes 13a and b) as well as secondary/secondary (Scheme 13c) diols. Also, the glycosidation of the NPG diol **73** (Scheme 13b) was both chemo and regioselective.

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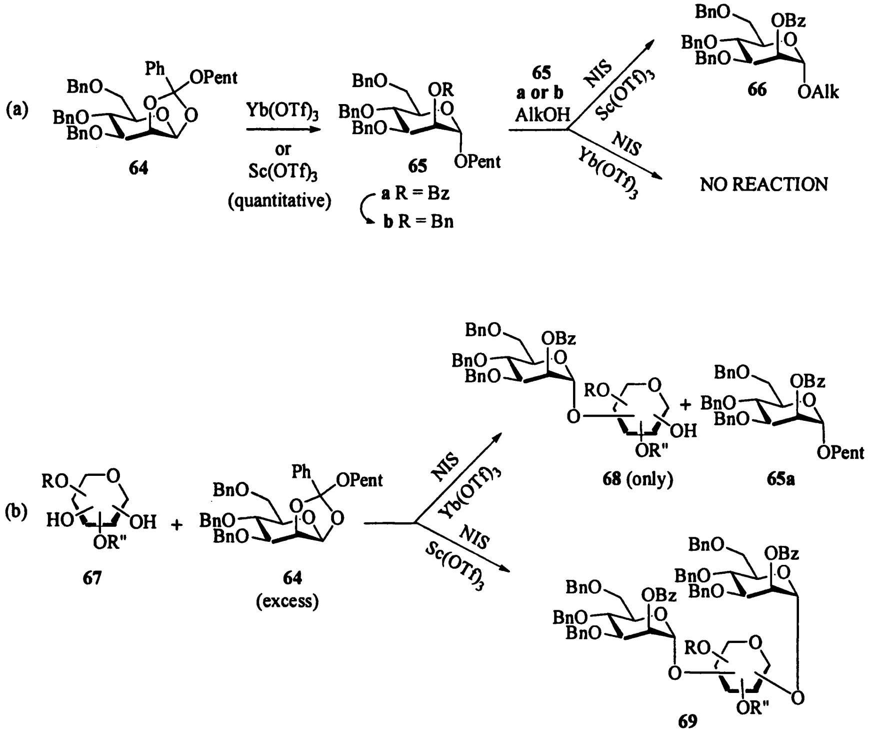
(a)
BnO
BnO
BnO
Ph
OPent
64
Yb(OTf)3
or
Sc(OTf)3
(quantitative)
OR
OPent
65
a R = Bz
b R = Bn
65
a or b
AlkOH
NIS
Sc(OTf)3
NIS
Yb(OTf)3
OBz
66
OAlk
NO REACTION
(b)
RO
HO
OH
OR"
67
64
(excess)
NIS
Yb(OTf)3
NIS
Sc(OTf)3
68 (only)
65a
69

Scheme 12. Activation of n*-pentenyl donors with* $Sc(OTf)_3$ *and* $Yb(OTf)_3$*.*

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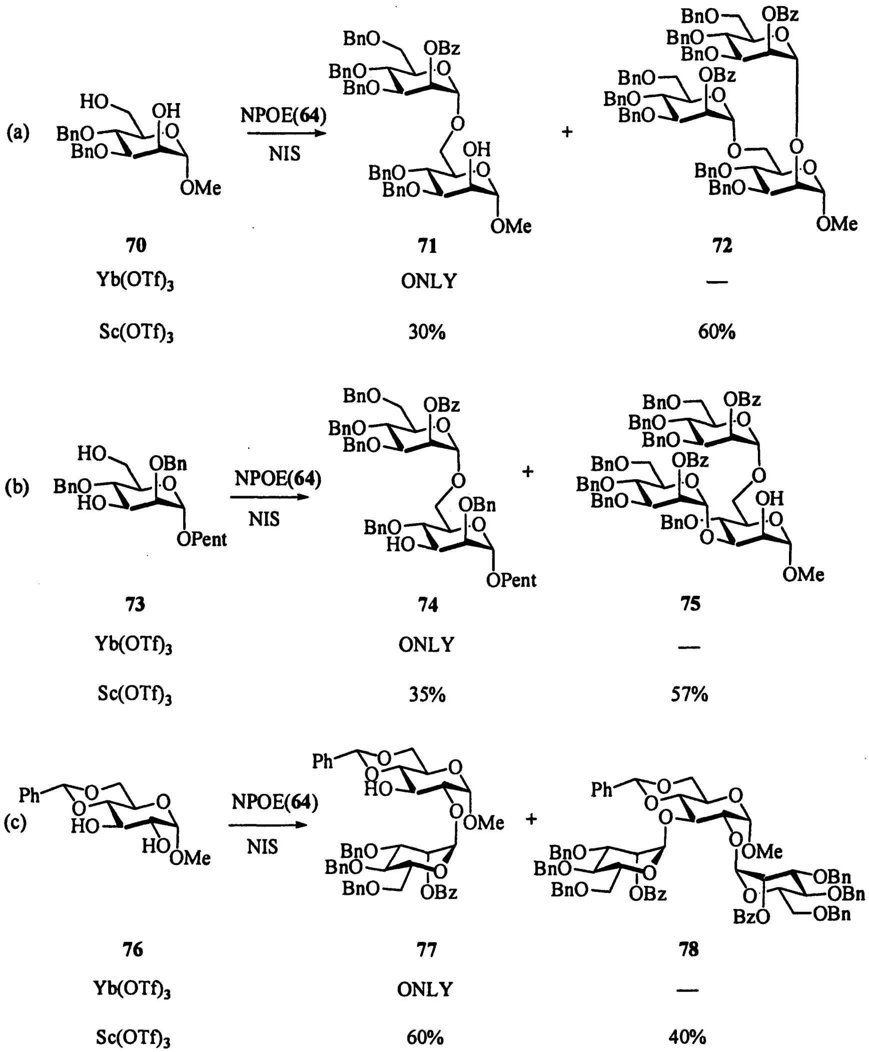

	70	71	72
Yb(OTf)$_3$		ONLY	—
Sc(OTf)$_3$		30%	60%

	73	74	75
Yb(OTf)$_3$		ONLY	—
Sc(OTf)$_3$		35%	57%

	76	77	78
Yb(OTf)$_3$		ONLY	—
Sc(OTf)$_3$		60%	40%

Scheme 13. The combination of NPOE/Yb(OTf)$_3$/NIS is exquisitely regioselective.

Gluco- and Galacto- donors

Attempts to extend the above excellent regioselectivities of the manno NPOE, **64** to its gluco and galacto counterparts **79** and **81** initially met with confusing failure. Several explanations for these differences are embedded in the data in Figure 1:

The disarmed manno model **34** is 4 k/cal **more** stable than its gluco/galacto counterpart **37**;
On the other hand, the gluco/galacto NPOE model **38** is ~2 kcal **less** stable than the manno counterpart **35**;
The combination of (a) and (b) implies that gluco/galacto donors should enjoy a substantial reactivity advantage over their manno counterparts.

Fortunately, being experimentalists, we had come to this conclusion from our failed experiments, and had crafted a solution that turned out to be consonant with the theoretical data.

Experimental Conditions

Typically, with the manno studies in Scheme 13, a solution containing the NPOE **64** and the diol acceptor was treated with NIS followed by addition of $Yb(OTf)_3$. However, when this procedure was used for gluco and galacto NPOEs, **79** and **81**, rapid rearrangement to the armed donors, **80** and **82**, occurred with the result that very little glycosidation took place.

Accordingly, in an alternative procedure, a solution containing the diol, NIS and $Yb(OTf)_3$ was treated with the gluco or galacto NPOE, **79** or **81**, added dropwise over 15 minutes. With this experimental change, regioselective glycosidation did occur, as seen in Scheme 14 (*32*). In general, the order of regioselectivity for several diols examined was *manno* > *galacto* > *gluco*.

Hydroxyl Glycosidation

Primary *versus* Secondary

Schemes 13 and 14 show the formation of six disaccharides resulting from regioselective glycosidations at the primary-OH of acceptor diols. It might

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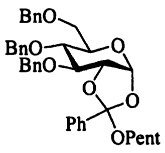
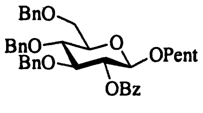
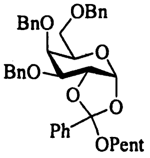

BnO
BnO
BnO
O
O
O
Ph
OPent

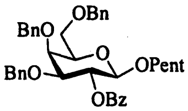
79


BnO
BnO
BnO
O
OPent
OBz

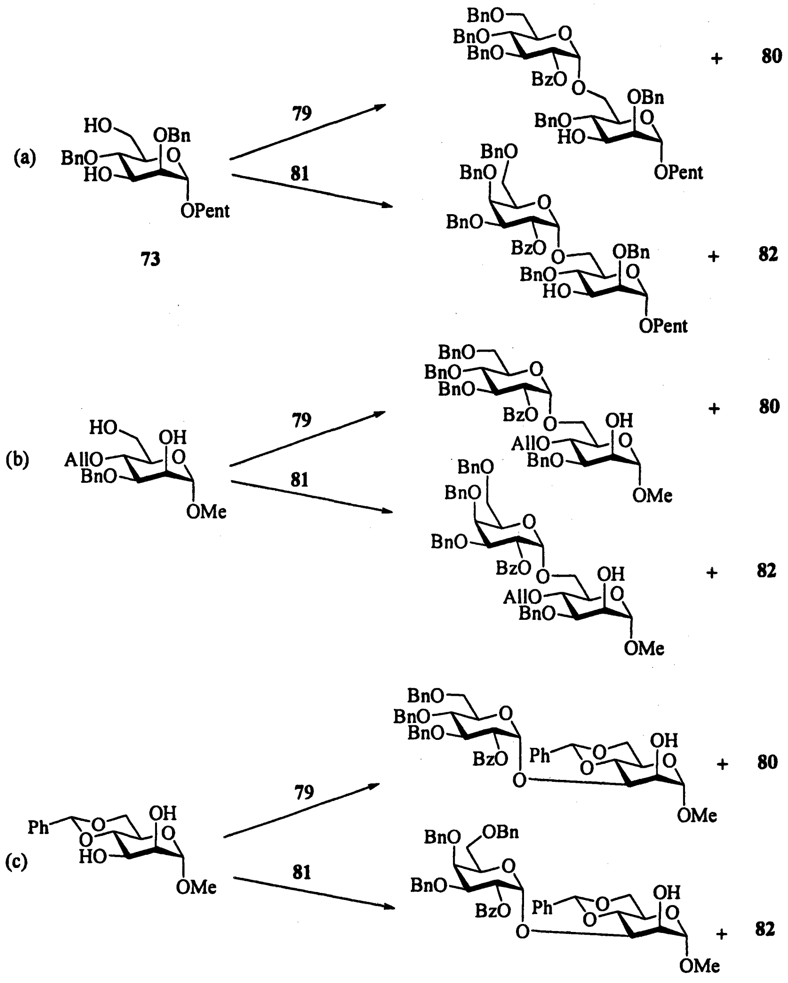
80


BnO
OBn
BnO
O
O
O
Ph
OPent

81


BnO
OBn
BnO
O
OPent
OBz

82


(a)
73
79
81
+ 80
+ 82
(b)
79
81
+ 80
+ 82
(c)
79
81
+ 80
+ 82

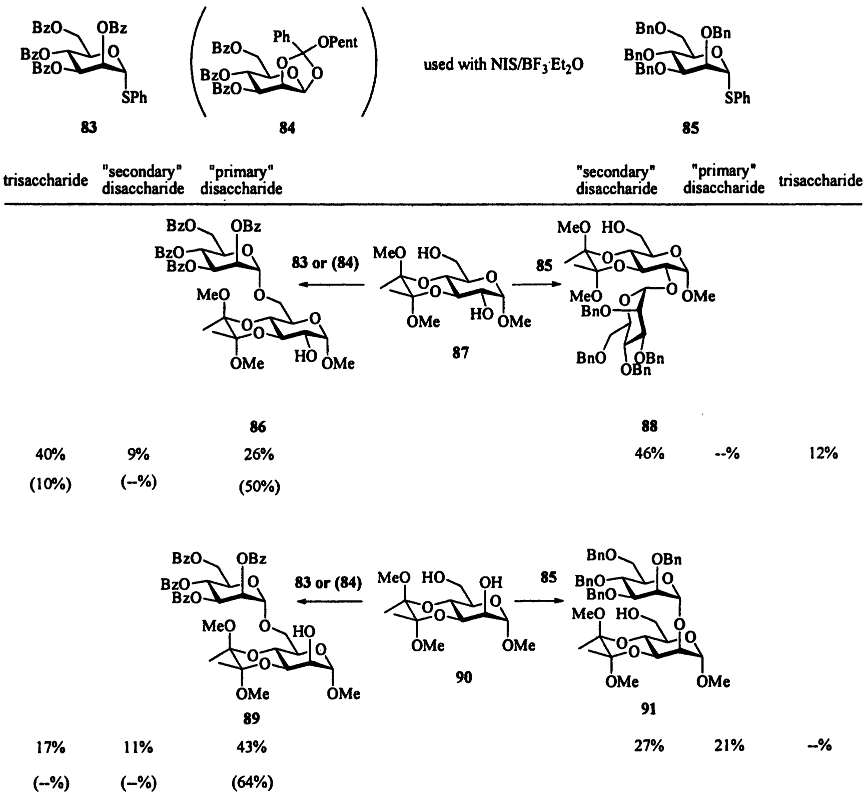
Scheme 14. Regioselectivity of Gluco *and* Galacto *NPOEs 79 and 81 with diols using NIS/Yb(OTf)$_3$.*

appear that these results simply reflect the classical primary-OH > secondary-OH reactivity that is accepted wisdom of organic chemistry (*33*). We therefore decided to put this fundamental tenet on trial by treating the "dispoke" gluco and manno diols **87** and **90** with the disarmed and armed thioglycosides **83** and **85**. The latter were used instead of the NPGs used above to test the generality of our findings.

The disarmed thioglycoside **83**, Scheme 15, shows that "primary" disaccharides **86** and **89** were the major products, the "secondary" disaccharides being formed in minor amounts, 9 and 11% respectively.

By contrast, with the armed donor **85** "secondary" disaccharides **88** and **91** were the major products, 46 and 27% respectively. Notably in the reaction with **88**, no "primary" disaccharide was detected.

The results in parentheses refer to NPOE **84**. They are seen to be even more selective for the primary-OH than with the disarmed counterpart **83**.


83
84
used with NIS/BF$_3$·Et$_2$O
85
trisaccharide
"secondary" disaccharide
"primary" disaccharide
"secondary" disaccharide
"primary" disaccharide
trisaccharide
83 or (84)
85
87
86
88
40%
(10%)
9%
(--%)
26%
(50%)
46%
--%
12%
83 or (84)
85
90
89
91
17%
(--%)
11%
(--%)
43%
(64%)
27%
21%
--%

Scheme 15. Glycosidation of primary/secondary diols with armed and disarmed donors 83 and 85 (and NPOE 84 shown in parentheses).

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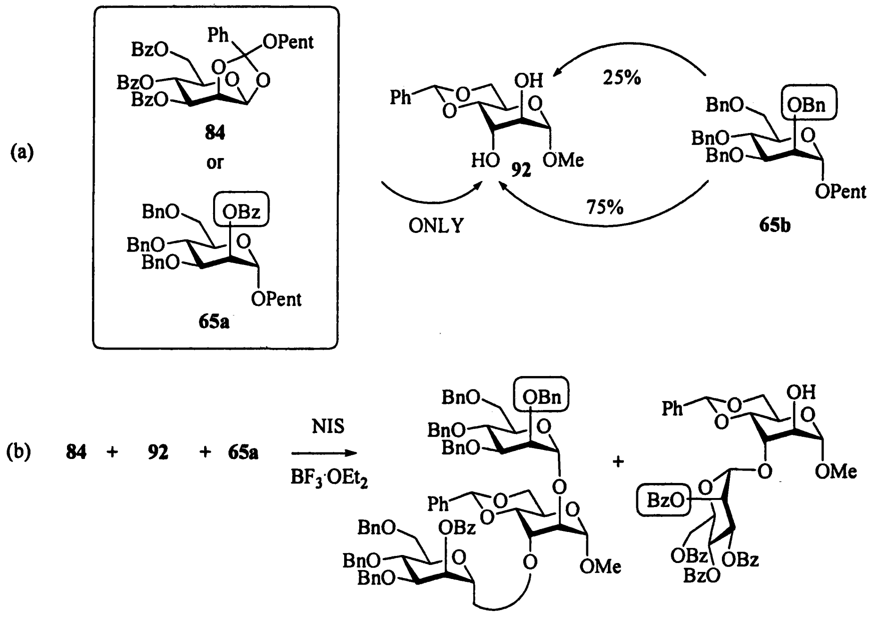
Hindered versus Non-Hindered

The studies in Scheme 15 indicate that selectivity between primary and secondary hydroxyls is donor dependent, NPOEs and disarmed donors favoring primary, while armed donors favor secondary.

The issue of hindered versus non-hindered hydroxyls has been addressed in a recent publication (*34*) in relation to the altroside **92**. As summarized in Scheme 16a, NPOE, **84**, or disarmed NPG, **65a**, reacted exclusively at the hindered C3-OH, with no reaction at the unhindered C2-OH. By contrast, the armed NPG **65b** went mainly for C3-OH (75%) but, significantly, it also went 25% for the C2-OH.


(a)
BzO
Ph
OPent
O
BzO
O
O
BzO
84
or
BnO
OBz
BnO
O
BnO
OPent
65a
Ph
O
OH
O
O
HO
92
OMe
25%
ONLY
75%
BnO
OBn
BnO
O
BnO
OPent
65b
(b)
84 + 92 + 65a
NIS
BF$_3$·OEt$_2$
BnO
OBn
BnO
O
BnO
O
Ph
O
O
BnO
OBz
O
BnO
O
O
OMe
BnO
+
Ph
O
OH
O
O
O
OMe
BzO
O
OBz
OBz
BzO

						93	94
(i)	1 equiv	+	1 equiv	+	1 equiv	27%	45%
(ii)	"	+	"	+	1.3 equiv	37%	37%
(iii)	"	+	"	+	1.6 equiv	43%	31%
(iv)	"	+	"	+	2 equiv	52%	19%
(v)	"	+	"	+	3 equiv	57%	16%

Scheme 16. Hindered versus *unhindered hydroxyls of altroside 92.*

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Reciprocal Donor Acceptor Selectivity (RDAS) and *in-situ*, Double Differential Glycosidations

The results in Schemes 15 and 16a suggest that there is a mutual preference of each donor for each-OH. For example, the secondary-OH of **87** or **90** chooses the armed donor, and *vice versa* i.e. the armed donor chooses the secondary-OH. In other words there is *Reciprocal Donor Acceptor Selectivity (RDAS).*

If *RDAS* is a valid concept, the two-component preferences in Schemes 15 and 16a should be maintained in three-component reactions where both donors are made to competitively glycosidate the hydroxyls of an acceptor diol. This strategy may be described as **in-situ, double differential glycosidation,** to distinguish it from "one-pot" procedures based on chemoselectivity (*35*) or orthogonal (*36*) principles.

A First Optimization Strategy

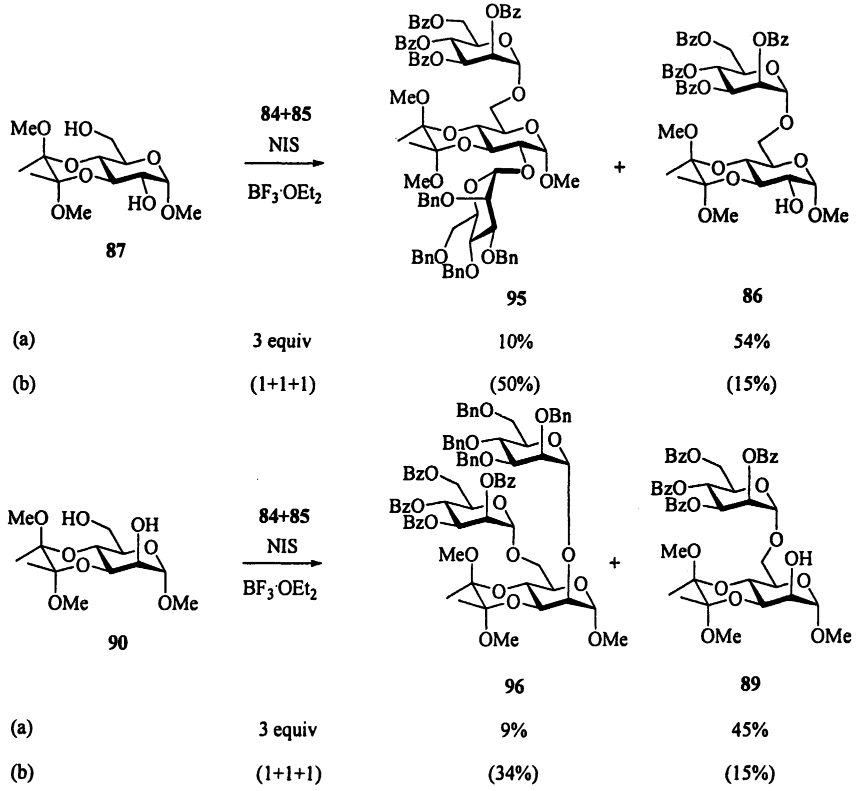
The RDAS concept has in fact been tested with the altroside **92** as shown in Scheme 16b (*37*). Thus, as seen in entry (i), a 1:1:1 mixture of NPOE **84**, diol **92,** and armed donor **65b**, gave rise to a single trisaccharide, **93**, wherein each donor had gone to its RDAS preferred-OH, this having been detremined in Scheme 16a. However, the major product was disaccharide **94**, which, from the summary in Scheme 16a, came from the NPOE, and NOT the armed donor **65b**.

In the hope of improving the yield of **93**, we employed the standard optimization practice of organic chemistry, by increasing the proportion of the armed donor **65b**, in the hope of glycosidating more of disaccharide **94**. Entries (ii) – (v) show that this practice was rewarded with a gradual increase in the yield of **93** and concomitant diminution in the amount of disaccharide **94**.

We consider it a striking confirmation of the RDAS principle, that even with an audacious 3:1 disparity of donors in Scheme 16, entry (v), there was no evidence of products from C3-OH glycosidation by 65b. This is made even more significant because in the two-component results (Scheme 16a), the C3-OH was preferred to the extent of 75%!!

A Second (Better!) Optimization Strategy

We wished to extend the RDAS concept to acceptor diols **87** and **90**, and for this, the NPOE **84** was used rather than disarmed donor **83**, since the former gave much higher yields of the "primary" disaccharides **86** and **89** (see Scheme



Scheme 17. Sequential NIS addition.

15). In the event, 1 equivalent of diol **87** or **90** was each presented with 1:1 amounts of NPOE **84** and armed donor **85** along with 3 equivalents of NIS. Gratifyingly, the results in Scheme 17a and c show that a single trisaccharide, **95** and **96** respectively, was produced in each case, each donor having gone to its RDAS preferred-OH, as had been found in Scheme 16b.

However, the 10% and 9% yields of **95** and **96** were again disappointing. The optimization strategy, applied in (ii)-(v) of Scheme 16 could have been tried again; but in spite of that success, it would be more elegant if the 1:1:1 ratio of reactants could be maintained, rather than resorting to the wasteful use of one reactant in excess.

A simple way to avoid this waste was in fact implicit in the **halonium ion transfer** concept in Figure 2. The success of this principle, as noted above, requires use of limited amounts of I^+, and addition of all 3 equivalents in one

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dose at the start of the reaction imperils that requirement. Accordingly, when the experiment in Scheme 17a was repeated, but with gradual addition of the 3 equivalents of NIS, the yield of trisaccharide **95** was raised from 10% to 50% with concomitant diminution in the amount of disaccharide **86** from 54% to 15% (Scheme 17b). Similarly, comparison of Schemes 17c and d shows increase in the yield of **96** from 9% to 34%, while the yield of **89** was decreased from 45% to 15% (*38*).

The rationale behind the improvements in Schemes 17b and d comes from the combination of two factors, *1)* the iodonium transfer between *n*-pentenyl and phenyl-1-thio-glycosyl donors, and *2)* the higher reactivity of orthoesters when compared to other armed or disarmed *n*-pentenyl or phenyl-1-thio glycosyl donors.

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Free-OHs on BOTH Donor and Acceptor

Synthesis of a Secondary-OH Pentasaccharide

In the above examples dealing with acceptor diols, the donors have been fully protected, in keeping with standard glycosidation practice. However the RDAS principle suggests that this may not (always) be necessary, and we have carried out some preliminary tests (*39*).

The dihydroxylated NPOE **97b** was an interesting candidate, because it is stable and easily prepared by silylation of the corresponding triol (*29b*). Reaction with the glucoside **98** proceeded to give **99a** from which triols **99b** and **99c** were obtained. These triols were chosen for study, because high mannose glycoproteins (*40*) tell us that C3-OH and C6-OH groups are nature's preferred glycosidation sites of mannosides.

We first tested for C3-OH glycosidation of triol **99b**. Treatment with diol NPOE **97b** followed by debenzoylation gave trisaccharide **100a** as the only coupling product in 50% yield. Iteration of the last two steps, gave tetrasaccharide **100b** in 40% yield.

Finally, glycosidation of acceptor tetrasaccharide heptaol **100b** with the diol NPOE donor **97b**, followed by debenzoylation gave pentasaccharide **101** as the only coupling product.

Synthesis of a Primary-OH Hexasaccharide

Encouraged by the results in Scheme 18, we proceeded to test triol **99c** for preparing a linear oligosaccharide. The results in Scheme 19 are equally

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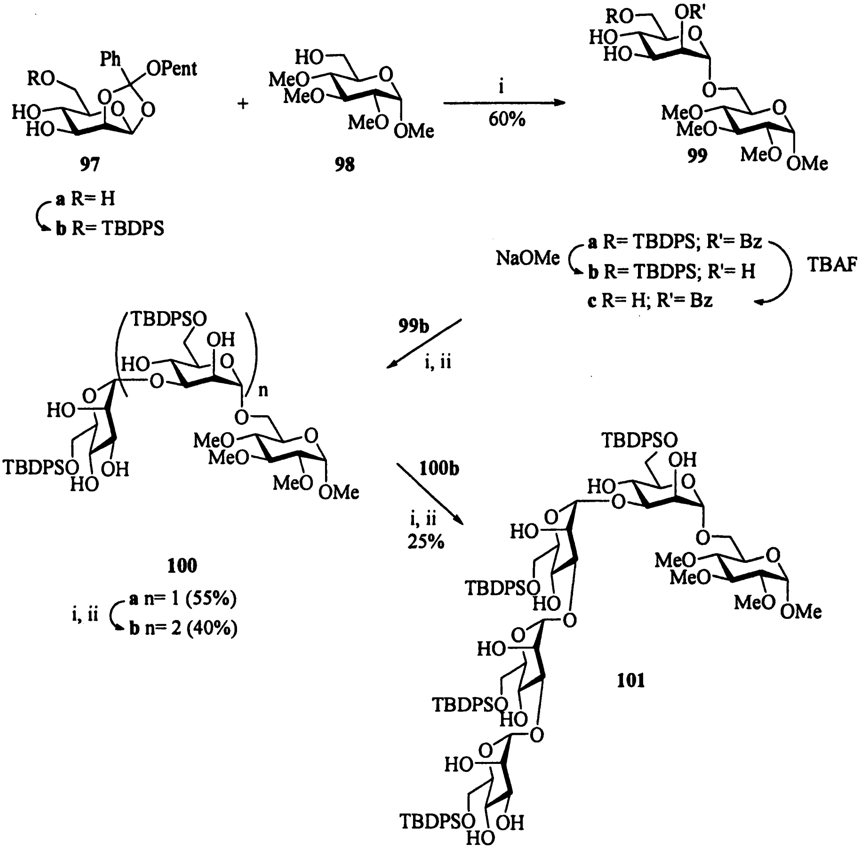
97
a R= H
b R= TBDPS
98
i
60%
99
a R= TBDPS; R'= Bz
b R= TBDPS; R'= H
c R= H; R'= Bz
NaOMe
TBAF
99b
i, ii
100b
i, ii
25%
100
i, ii
a n= 1 (55%)
b n= 2 (40%)
101
TBDPSO
OPent
OMe

Reagents and conditions: i, NIS, $BF_3 \cdot Et_2O$; ii, NaOMe, MeOH

Scheme 18. Synthesis of a Secondary-OH Pentasaccharide with Acceptor and Donor Polyols.

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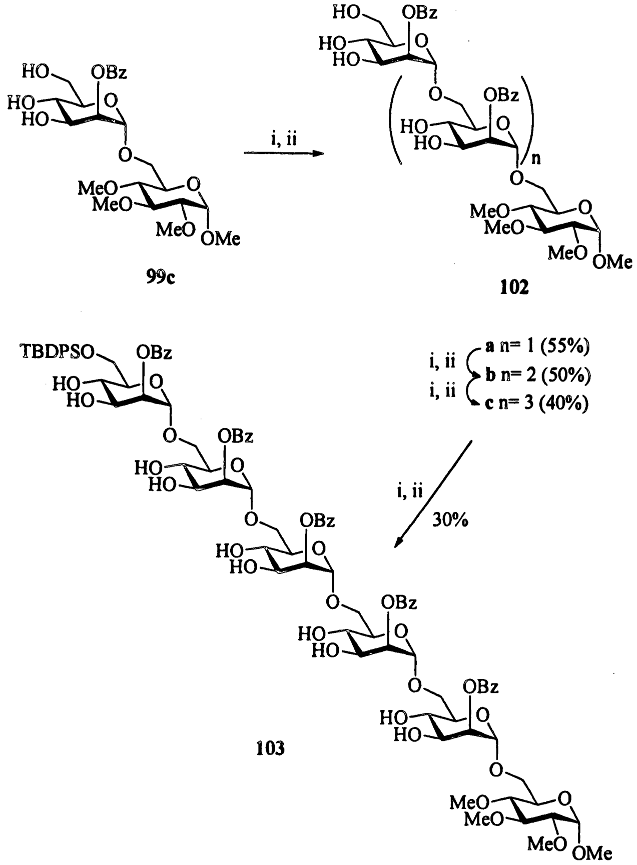
i, ii
99c
102
n
i, ii
i, ii
a n= 1 (55%)
b n= 2 (50%)
c n= 3 (40%)
i, ii
30%
103
TBDPSO
OBz
HO
MeO
OMe

Reagents and conditions: i, NIS, $BF_3{\cdot}Et_2O$; ii, TBAF, THF

Scheme 19. Synthesis of a Primary-OH Hexasaccharide with Acceptor and Donor Polyols.

encouraging. Thus iterative elaboration of **99c** proceeded to give good yields of higher saccharides **102a, 102b** and **102c**. Finally acceptor pentasaccharide **102c,** comprising NINE free-OH groups reacted with the NPOE donor diol **97b** to give hexasaccharide **103** in 30% yield **as the only identifiable coupling product.**

Summary

Protecting groups are a necessary evil, and their installation and removal are the most time-consuming aspects of oligosaccharide synthesis regardless of the modality, solution, solid-phase, automated or programmed. Minimizing their use is therefore a worthwhile objective. The concept of donor/acceptor "match" provides one solution, since there is no need to protect an hydroxyl that does not match the donor being used. *n*-Pentenylorthoester donors are capable of exquisite regioselectivity, whereby the matching-OH in a polyol acceptor can be identified. On the basis of such preliminary analyses, in situ, double differential glycosidations involving two donors and a diol acceptor can be carried out.

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Chapter 8

Synthesis of N-5-Derivatives of Neuraminic Acid and Their Application as Sialosyl Donors

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As terminal constituents of glycoconjugates, sialic acids are involved in a wide range of biological phenomena. Different approaches and strategies have been developed to improve yield and stereoselectivity in sialylation reactions. Recently it has been reported that a substituent at the C-5 amino group in sialic acid influences the reactivity of both sialyl donors and acceptors. This review summarizes the latest accomplishments in sialylations using different N-substitued sialyl derivatives.

Sialic acids are a diverse family of more than 40 naturally occurring 2-keto-3-deoxy-nononic acids amongst which *N*-Acetylneuraminic acid (Neu5Ac), *N*-glycolylneuraminic acid (Neu5Gc) and *N*-acetyl-9-O-acetylneuraminic acid (Neu5,9Ac$_2$) are the three most widespread (Figure 1).[1,2]

In particular, Neu5Ac is ubiquitous, while the others are not found in all species. Although free sialic acid can be detected in several types of human body fluids, it is mainly present as a component of the oligosaccharide chain in glycoconjugates (glycoproteins and glycolipids). In the cell, glycoconjugates are synthesized in the Golgi apparatus then transported in the cell membrane where they are involved in a wide variety of biological phenomena from cell growth and differentiation to cell adhesion.

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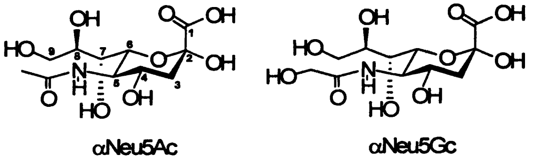
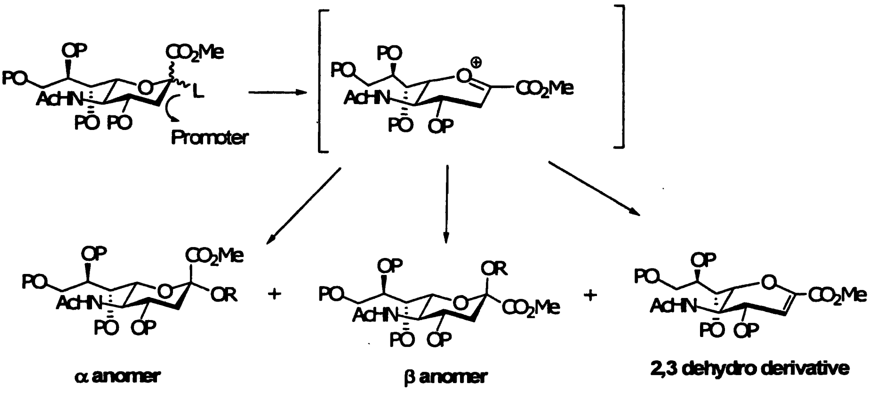


Figure 1.

In addition, being in such exposed position, glycoconjugates are also the receptors for bacteria and viruses. Thus, it is not surprising that, found at the terminal position of the glycoconjugate chain, sialic acid is directly correlated to numerous biological phenomena. In N-linked glycoproteins, Neu5Ac appear essentially as terminal sugars, α(2→3) or α(2→6)-linked to galactosides or α(2→6)-linked to N-acetyl-galactosaminides {e.a. Neu5Acα(2→3)Gal, Neu5Acα(2→6)Gal and Neu5Acα(2→6)GalNAc} whereas in O-linked glycoproteins, often terminal Neu5Acα(2→6)GalNAc moieties can be found. The disialosyl structures Neu5Acα(2→8)Neu5Ac and Neu5Acα(2→9)Neu5Ac have also been found as constituents of glycoproteins and glycolipids.[1,3-6]

Although extensively explored, the chemical synthesis of sialosides in high yield with complete stereoselectivity is still a notable challenge. The presence of a destabilizing electron-withdrawing carboxylic group together with a tertiary anomeric center and the lack of a participating auxiliary often drive glycosylation reactions toward competitive elimination reactions resulting in poor stereoselectivity (β anomer) and in the formation of a 2,3 dehydro derivative (Scheme 1).

In addition, Neu5Ac is also a poor glycosyl acceptor for the synthesis of α–linked (2-8) dimers, due to the intramolecular hydrogen bonds between 8-OH, the acetamido and/or the carboxylate group (Figure 2).



Scheme 1.

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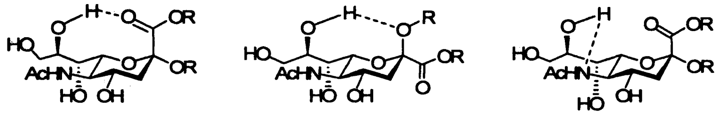

Figure 2.

To address the problems associated with chemical sialylation, different strategies have been developed in the past few years, in particular focusing on the nature of the leaving group or promoter as well as the introduction of participating auxiliaries at the C-3 positions (indirect methods).[7-9] Recently, modifications of the C-1 carboxylic group[10,11] as well as the C-5 acetamido group have been reported as alternative strategies to optimize glycosylation reactions. In particular, it has been shown that the nature of the protecting group at the C-5 position plays an important role in controlling yield and stereoselectivity in glycosylation reactions.

This review summarizes the latest developments in the chemistry of sialic acid which involve modifications of the natural 5-acetamido function (NHAc) into a) N-acetylacetamido (NAc_2), b) azido (N_3), c) N-trifluoroacetamido (NHTFA) and d) N-trichloroethoxycarbonyl (NHTroc). Last section is dedicated to protecting groups that have been introduced at the N-position but their potential as sialosyl donors has not emerged yet, *e.g.* tert-Butoxycarbonyl (Boc), Benzyloxycarbonyl (Z or CBz) and 9-Fluorenylmethoxycarbonyl (Fmoc).

a) N-Acetylacetamido (NAc_2)

Boons et al.[12] reported that an additional acetyl group in methyl-thio-5-N-acetylacetamido derivative **1** dramatically increases the reactivity of the donor when compared to the corresponding mono-N-acetylated **2**. For example, an NIS/TfOH-promoted coupling of 2-(trimethylsilyl)ethyl 6-*O*-benzoyl-β-D-galactopyranoside **3** with sialyl donor **1** yielded the desired α(2→3)-linked disaccharide **4** in a yield of 72% in less than 5 min, while the corresponding mono-N-acetylated derivative required more reaction time (2-6 h) and a higher number of equivalents of sialosyl donor in respect to glycosyl acceptor to achieve similar results (Scheme 2). Higher yield (81%) could be obtained when a 4,6-*O*-benzylidene protected galactoside was used as a glycosyl acceptor.[12] The improved glycosyl donor properties of **1** were rationalized by the observation that protection of the *C*-5 acetamido group with a strong electron withdrawing amino protecting group would reduce its nucleophilicity thereby minimizing possible side reactions in glycosylations. The additional acetyl group at the C-5

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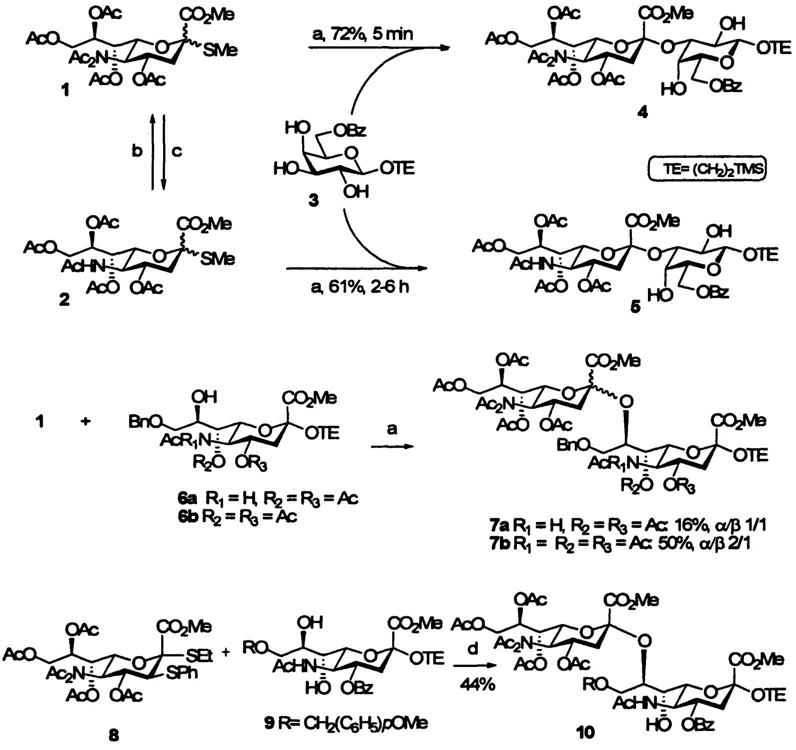
position is easily introduced from the N-monoacetylated donor or directly from the deprotected Neu5Ac with concomitant O-acetylation and it is removed under Zémplen deacetylation conditions with concomitant *O*-acetyl group removal. A convenient feature of this approach is that neither N-acetylation nor N-deacetylation requires additional protecting group manipulations.



Scheme 2. Reaction conditions: a: NIS/TfOH/MS 3Å/MeCN, -40°C; b: $CH_2=C(CH_3)OAc/TsOH$; c: i) MeONa/MeOH; ii) Ac_2O/Py d: $AgO_3SCF_3/MeSBr/$ MS 3Å/MeCN, CH_2Cl_2, -45°C

The higher reactivity of the sialyl donor bearing an NAc_2 moiety has also been proven for the synthesis of the α(2-8) dimer. As mentioned above, the synthesis of α(2-8) glycosidic bond is complicated by the low reactivity of the acceptor due to intramolecular hydrogen bonds (Figure 2). Thus, 5-N-diacetyl neuraminyl derivative **1** gave the dimer **7a** as an anomeric mixture in modest yield (16%). This result should be compared with similar sialylation using the N-monoacetylated donor which gave the corresponding dimer in 5% yield.[13]

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On the other hand, when sialosyl acceptor **6b** which bears a 5-N-acetylacetamido group was used, the desired dimer **7b** was obtained in an improved yield (50%) and stereoselectivity (α/β=2:1).[14] This finding suggests a crucial role of the N-5 protecting group in the control of the reactivity of 8-OH, probably due to the decrease of H-bonding between the 5-N and the 8-OH.

These promising results encouraged the development of different approaches where 5-N-diacetyl donors modified at C-3 have been tested.[15,16] For example, ethyl-thio-3-thiophenyl-5-N-acetylacetamido donor **8** proved to be more reactive than the corresponding monoacetylated donor with a series of different glycosyl acceptors. In fact, sialylation of **8** with acceptor **9** gave the dimer **10** in 44% yield, whereas the corresponding monoacetylated counterpart gave the corresponding dimer in 28% yield (Scheme 2).[16] In both cases complete α-stereoselectivity was achieved.

b) Azido (N_3)

The synthesis of 5-N-azido derivatives of N-acetylneuraminic acid has been accomplished by both enzymatic[17-21] and chemical methods.[22-25] In particular, Schmidt[23] and Wong[24] described the introduction of the azido group from known sialosyl donors **11a** and **11b** respectively by using trifluoromethanesulfonyl azide (triflic azide, TfN_3) as the diazo transferring reagent (Scheme 3).

Similarly, sialosyl acceptors bearing an azido moiety at C-5 were synthesized to reduce the unfavorable hydrogen bonds for the synthesis of α(2-8) and α(2-9) dimers. Thus, 2-thiocresol sialyl donor **12b** gave significantly improved α-stereoselectivity in sialylation, especially when applied to the synthesis of (2-9)-linked dimers. For example, glycosylation of **12b** with acceptor **13b** gave the desired α-linked dimer with complete stereoselectivity in 65% yield. Under the same reaction conditions, the corresponding N-acetyl and N-acetylacetamido donors gave the desired disaccharide as α,β-anomeric mixtures (3:1 and 4:5, respectively). Chemoselective activation of 5-azido phosphite **15** over thioglycoside acceptor **16**, which also bears an azido group at C-5, allowed the stereoselective synthesis of dimer **17** in 51% yield.[24] Thus, **17** can be used directly in subsequent sialylation.

The high α-stereoselectivity observed was attributed to both steric and electronic effects. The azido group is electron-withdrawing and linear, therefore, it can stabilize the acetonitrilium intermediate and allow an easier access of the acceptor from the α face.[24] 5-azido donors and acceptors are notably more reactive than the common acetamido group containing derivatives (Scheme 3).[24,26] However, the azido group's control on yield and stereoselectivity in

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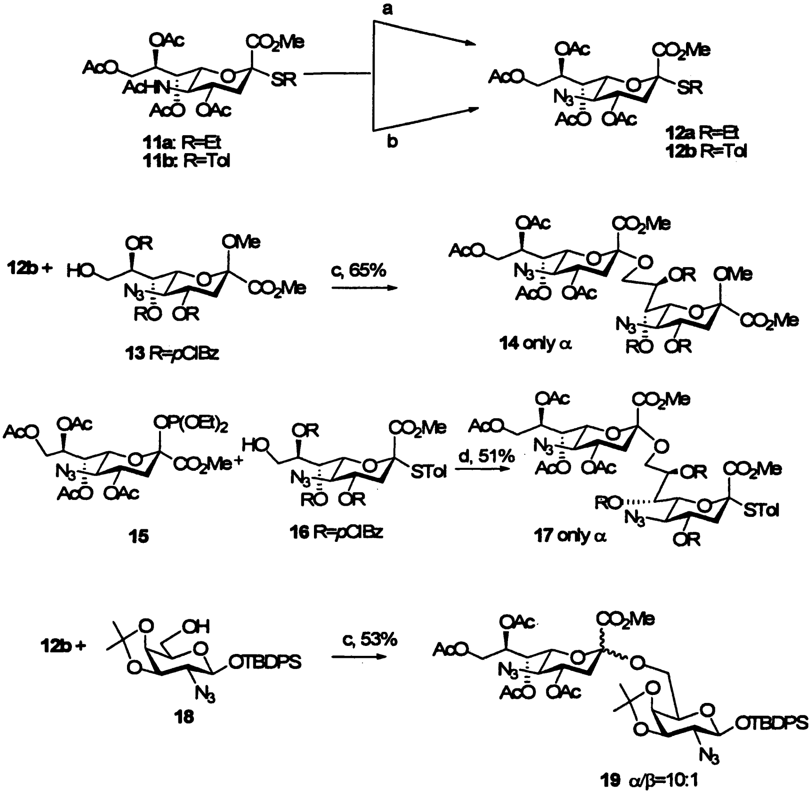
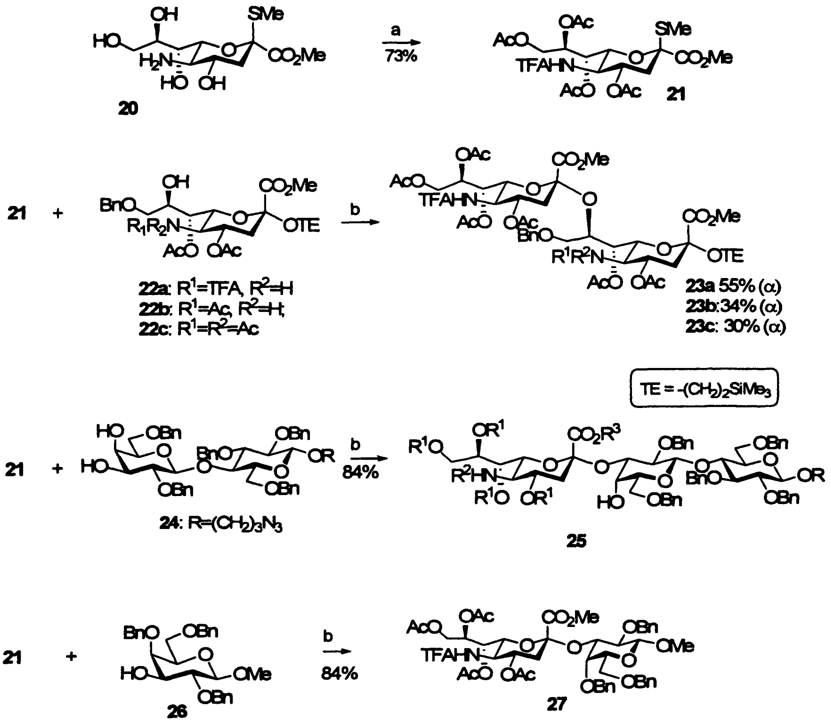

a
b
11a: R=Et
11b: R=Tol
12a R=Et
12b R=Tol
12b +
c, 65%
13 R=pClBz
14 only α
15
16 R=pClBz
d, 51%
17 only α
12b +
c, 53%
18
19 α/β=10:1

Scheme 3. Reaction Conditions a (11a): i)KOH/EtOH; ii) TfN$_3$/DMAP; iii) Ac$_2$O/Py; iv) CH$_2$N$_2$, 63% overall yield; b (11b): i) MsOH/MeOH, 55%; ii) TfN$_3$/CuSO$_4$ 5H$_2$O, MeOH; iii) Ac$_2$O/Py, 66% over two steps; c: NIS/TfOH/MS 3Å/MeCN, -40 °C; d: TMSOTf/MS 3Å/MeCN, -40 °C

glycosylation reactions is less prominent with sterically hindered and/or less reactive acceptors, such for the synthesis of α(2-3) galactosyl derivatives and α(2-8) dimers. For the latter, the coupling between 5-azido perbenzylated thioglycoside donor and 5-azido sialyl acceptor did not give any dimeric product.[27]

c) Trifluoroacetamido (N-TFA)

As mentioned above, it has been postulated for the N-acetylacetamido derivatives that reducing the nucleophilicity/basicity of the C-5 amino group results in an increased reactivity of sialyl donor and acceptors. Hence, a potent electron-withdrawing group such as trifluoroacetamido should help to further decrease the unfavorable H-bonds between the acetamido function and 8-OH.



Scheme 4. Reaction Conditions a: i) CF_3COOMe/Et_3N, MeOH ii) Ac_2O/Pyridine; b: NIS/TfOH/MS3Å, MeCN, -35°C

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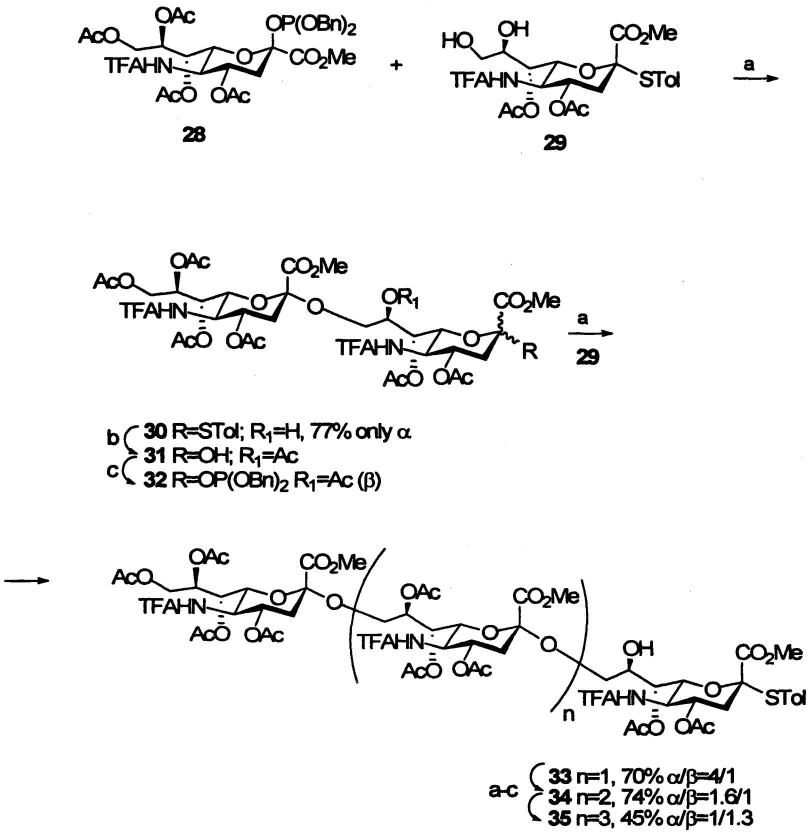
The introduction of a trifluoroacetyl group can be accomplished by reaction of the common precursor **20** with methyltrifluoroacetate in the presence of triethylamine and methanol (Scheme 4).[28] Thus, a 5-N-trifluoracetyl sialyl donor **21** and acceptor **22a** proved to be far more reactive than the corresponding N-acetamido **22b** and N-acetylacetamido **22c** derivative (Scheme 4).[29]

While complete stereoselectivity was observed in every case, and the highest yields were achieved with 5-N-TFA acceptor **22a**. The versatility of the N-TFA donor allowed a highly efficient synthesis of the human melanoma associated antigen GD_3 derivative, which has multiple 2-8-linked Neu5Ac residues. To date, N-TFA protected sialylations are the only examples of stereoselective synthesis of α(2-8) dimers by direct methods.[29-31]



Scheme 5. Reaction Conditions a: TMSOTf/ MS3Å/MeCN/-40°C b: i) Ac_2O/pyridine; ii)NBS, acetone/H_2O; c: 1H-tetrazole, dibenzyl N,N-diisopropylphosphoramidite, MeCN

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High reactivity of 5-N-TFA sialyl donor has been reported also for the synthesis of α(2-3)- and α(2-6)-linked derivatives with a wide range of galactosyl acceptors.[32] Thus, complete stereoselectivity and the best yields were accomplished for more sterically hindered/less reactive acceptors (e.g. **24** and **26**). The reactivity of N-TFA donor is so high that in case of primary hydroxyl groups or triols, loss of stereoselectivity and/or regioselectivity was observed.

Recently, phosphite sialosyl donors bearing a C-5 trifluroacetamido group were applied to the synthesis of α(2-9) oligomers (Scheme 5).[33]

Also in this case, these donors gave a higher degree of α-anomeric selectivity as compared to conventional sialyl donors. Thus, N-TFA phosphite donor **28** was coupled with N-TFA thioglycoside acceptor **29** thereby obtaining the α-linked dimer **30** with 77% yield. The STol moiety of **30** was then replaced with phosphite. By iterating these coupling-reprotection steps, higher-order sialosides were obtained with high yield and stereoselectivity.

d) Trichloroethoxycarbonyl (N-Troc)

Conversion of the N-acetyl phenylthioglycoside derivative **36** into the corresponding N- trichloroethoxycarbonyl (Troc) derivative **37** was described by Wu[34] and Kiso[35] using succinimidyl 2,2,2-trichloroethyl carbonate and trichloroethylchloroformate, respectively (Scheme 6).

Kiso[35] proved that sialyl donor **37** is more reactive than the corresponding N-monoacetylated derivative **36** when coupled with acceptor **38**. However, loss in stereoselectivity was also observed (Scheme 6).

For the synthesis of (2-9) dimer, armed thiosialoside donor **37** was coupled with disarmed thiosialoside acceptor **41** which bears the common acetamido group at C-5. The resulting disaccharide **42** was obtained in moderate yield and stereoselectivity. Interestingly, the arming and disarming of these building blocks was achieved from a remote position.

Recently, Takahashi[36] reported the use of **37** for the synthesis of 2,6-sialyl T antigen by one-pot glycosylation with acceptor **43** and subsequent addition of donor **44**. The desired trisaccharide **45** was obtain in 77% overall yield (Scheme 7).

Miscellaneous Substituents

Modifications of the conventional N-5 acetyl group into N-tert-butyloxycarbonyl (Boc),[37] N-benzyloxycarbonyl (Cbz, Z),[38] N-phthalimido

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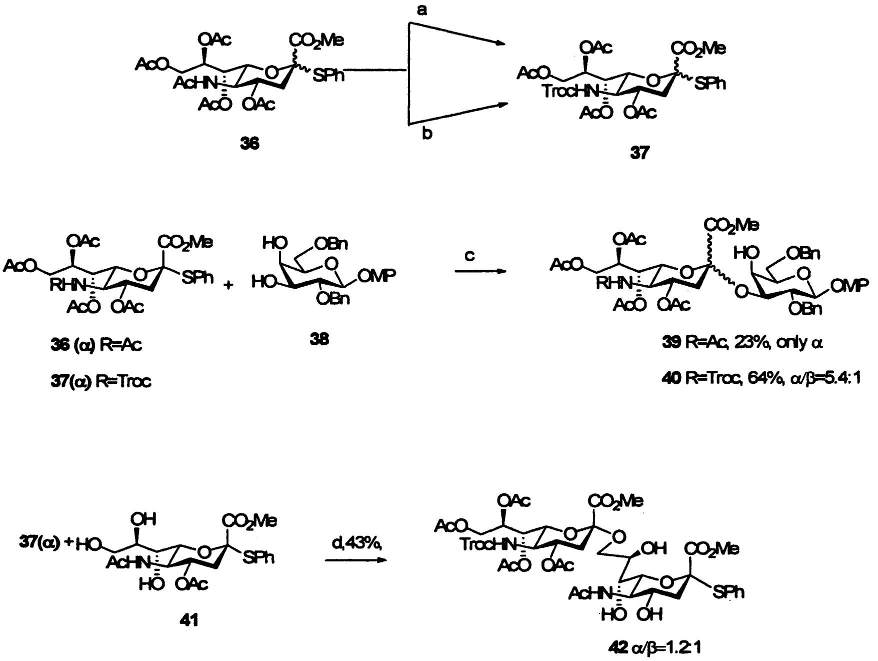
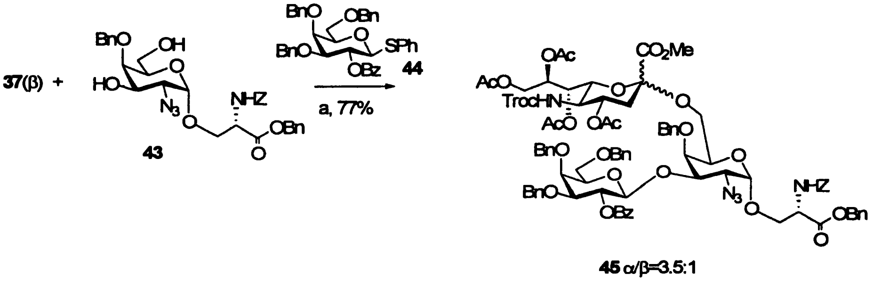

a
b
c
d,43%,
OAc
CO2Me
AcO
AcHN
O
SPh
AcO OAc
36
TrocHN
37
HO
OBn
OMP
RHN
36 (α) R=Ac
37(α) R=Troc
38
39 R=Ac, 23%, only α
40 R=Troc, 64%, α/β=5.4:1
37(α) +
OH
HO OAc
41
42 α/β=1.2:1

*Scheme 6. Reaction Conditions a:i)MsOH, MeOH, ii)TrocSu, NaHCO$_3$, 77% (2 steps);iii) Ac$_2$O, Py, 94%; b: i) i)MsOH, MeOH; ii)TrocCl, Et$_3$N, MeOH; iii)Ac$_2$O, Py,68% (three steps); c: NIS, TfOH, MS3Å, for **36**: MeCN-CH$_2$Cl$_2$, -25°C; for 37: EtCN, -50°C; d: : NIS, TESOTf, MS3Å, MeCN-CH$_2$Cl$_2$, -40°C*


37(β) +
BnO
OH
HO
N3
NHZ
OBn
O
43
BnO
OBn
SPh
OBz
44
a, 77%
OAc
CO2Me
AcO
TrocHN
AcO OAc
45 α/β=3.5:1

Scheme 7. Reaction Conditions a:37, 43, NIS, TfOH, MS3Å, MeCN, -35°C; then 44, NIS, TfOH, CH$_2$Cl$_2$, -30°C

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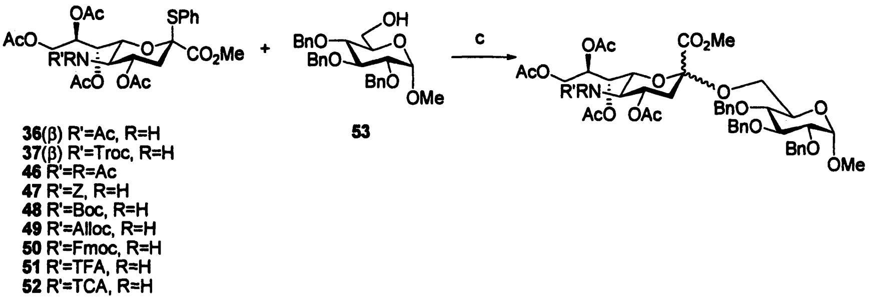
(Phth),[39] and N-*tert*-butyloxycarbonylacetamido[40] have been reported as alternative strategies to allow a simplified access to the C-5 free amino group upon deprotection. However, their influence in the reactivity or stereoselectivity in the syntheses of sialosides has not yet emerged. Recently, Takahashi reported a comparative study on the reactivity and stereoselectivity in glycosylation reactions of differently N-protected thioglycosides when coupled with perbenzylated methylglucoside **53** as acceptor (Scheme 8 and Table 1).[36]


OAc
SPh
AcO
R'RN
O
CO2Me
AcO
OAc
+
OH
BnO
O
BnO
BnO
OMe
c
OAc
CO2Me
AcO
R'RN
O
O
AcO
OAc
BnO
O
BnO
BnO
OMe
36(β) R'=Ac, R=H
37(β) R'=Troc, R=H
46 R'=R=Ac
47 R'=Z, R=H
48 R'=Boc, R=H
49 R'=Alloc, R=H
50 R'=Fmoc, R=H
51 R'=TFA, R=H
52 R'=TCA, R=H
53

Scheme 8. Reaction Conditions a: NIS, TfOH, MS3Å, , MeCN

Table 1.

Entry	Donor	R	R'	Yield %	α:β ratio
1	36 β	H	Ac	47	6:1
2	37 β	H	Troc	91	8:1
3	46	Ac	Ac	65	2:1
4	47	H	Z	68	5:1
5	48	H	Boc	-	-
6	49	H	Alloc	44	7:1
7	50	H	Fmoc	91	6:1
8	51	H	TFA	92	11:1
9	52	H	TCA	83	10:1

Based on these results, sialosyl donors bearing N-Troc (**37**, entry 2), N-TFA (**51**, entry 8) and N-trichloroacetate (**52**, entry 9) gave the best yields and stereoselectivity.

Conclusions

Although it is clear that a modification at the 5-N position of N-acetylneuraminic acid influences reactivity and stereoselectivity in glycosylation reactions, a universal 5-N protecting group that would address all the challenges of sialylation has not yet emerged.

N-Acetylacetamido can be obtained directly from the acetamido derivative and offers the advantage of being introduced and removed under mild conditions. High yields and stereoselectivities can be accomplished with less reactive acceptors by using the trifluoroacetamido group, while the azido group can be a suitable choice when the coupling with primary alcohols is desired. Trichloroethoxycarbonyl allows a convergent armed-disarmed approach for the synthesis of (2-9) dimers, but in general gives lower yields and stereoselectivity than the azido and trifluoroacetamido approaches (Table 2).

Table 2.

Protecting Group	Advantages	Suitable for
Ac_2	Introduction does not require amino deprotection; removal by Zemplen deacetylation	Primary alcohols
N_3	Stable in acidic and basic conditions, can be removed in the presence of acetyl groups	Primary alcohols
TFA	High yields and stereoselectivities observed with a wide range of acceptors	Hindered, less reactive alcohols
Troc	Can be removed in the presence of acyl groups and acid/base labile groups, such as amino acids	Armed-disarmed approach

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Chapter 9

2'-Carboxybenzyl Glycosides: A Novel Type of Glycosyl Donors and Their Application to Oligosaccharide Synthesis

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In this chapter, we illustrate our contribution to the efficient and stereocontrolled glycosylation method employing 2′-carboxybenzyl (CB) glycosides as glycosyl donors. The CB glycoside method has been found to be a powerful tool for the stereoselective β-mannopyranosylation, 2-deoxyglycosylation, and β-arabinofuranosylation. Furthermore, the CB glycoside method has been successfully applied to the efficient construction of oligosaccharides by the latent (BCB)-active (CB) glycosylation strategy.

The development of efficient and stereoselective glycosylation methodologies has attracted a great deal of attention in the past a decade due to the explosive growth of important biological functions of complex oligosaccharides and glycoconjugates in glycobiology (*1*). Devising new glycosyl donors and developing new activation systems for existing donors have led to major advances in this field. For example, several glycosylation methodologies, based on quite efficient glycosyl donors such as thioglycosides (*2*), glycosyl sulfoxides (*3*), glycals (*4*), glycosyl trichloroacetimidates (*5*), *n*-pentenyl glycosides (*6*), and glycosyl fluorides (*7*), have been available. Recently, glycosylation methods using new glycosyl donors and employing new activation systems for existing glycosyl donors have been reported (*8*). However,

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there still remains a need for more efficient and generally applicable new glycosylation methodology.

In this chapter we introduce 2′-carboxybenzyl (CB) glycosides **B** (Figure 1) as a novel type of glycosyl donors that is useful for the stereoselective β-mannopyranosylation (*9*), 2-deoxyglycosylation (*10*), β-arabinofuranosylation (*11*), but also very effective for the construction of oligosaccharides by the latent-active glycosylation strategy (*11*, *12*). The CB glycoside **B** was prepared from 2′-(benzyloxycarbonyl)benzyl (BCB) glycoside **A** by the selective removal of its benzyl ester functionality. Treatment of **B** with triflic anhydride followed by spontaneous lactonization of the resulting glycosyl triflate **C** would afford the oxocarbenium ion **D** by extrusion of stable phthalide. Reaction of **D** with the glycosyl acceptor (Sugar-OH) would give desired glycoside **E**.

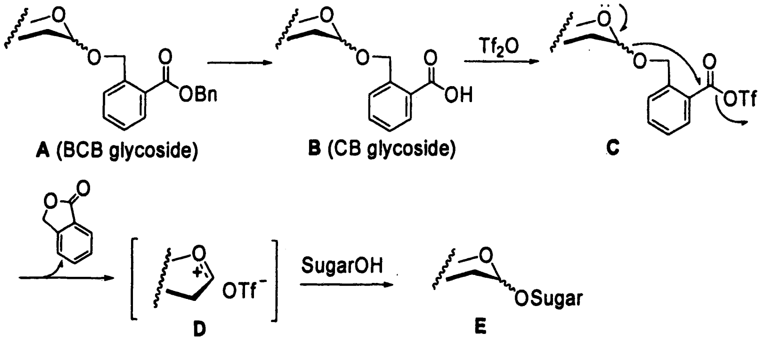

Figure 1.

1. β-D-Mannosylation employing CB glycoside method

The stereospecific formation of the 1,2-β-D-mannopyranosyl linkage has been one of the challenges in the glycoside synthesis. Although several strategies for the β-mannopyranosylation (*13*) have been developed including Crich's recent approach (*14*) using 4,6-*O*-benzylidene protected glycosyl sulfoxides or thioglycosides as glycosyl donors, the construction of β-mannopyranosyl linkages still poses a great challenge.

The protected CB mannopyranoside **5** was efficiently prepared starting from tetraacetylmannosyl bromide (**2**) and benzyl 2-(hydroxymethyl)benzoate (**1**), which was readily obtained in large quantities from inexpensive phthalide, as shown in Scheme 1. Coupling of **2** and **1** followed by deacetylation of the resulting tetraacetylmannoside afforded BCB mannoside **3**, of which benzylidenation and subsequent benzylation provided protected BCB mannoside

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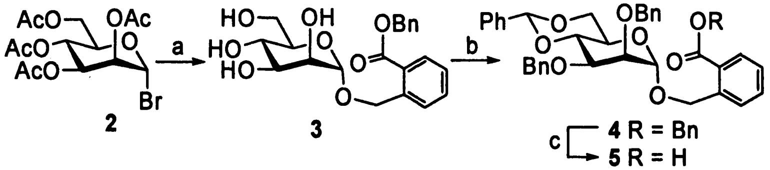
4. Selective hydrogenolysis of the benzyl ester functionality in **4** was readily achieved in the presence of ammonium acetate (*15*) to afford the desired CB mannoside **5**, which could be stored at room temperature for a few months without any change.

Scheme 1[a]



[a](a) (i) benzyl 2-(hydroxymethyl)benzoate (**1**), $HgBr_2$, $Hg(CN)_2$, CH_3CN, 86%; (ii) NaOMe, MeOH, 93%; (b) (i) $PhCH(OMe)_2$, CSA, DMF, 70%; (ii) BnBr, NaH, DMF, 80%; (c) H_2, Pd/C, NH_4OAc, MeOH, 95%.

Glycosylations of various acceptors with the donor **5** were carried out by dropwise addition of 2 equiv of glycosyl acceptors to the solution of 1 equiv of **5** and 2 equiv of DTBMP in the presence of 4Å molecular sieves at –78 °C. Glycosylations of primary alcohol acceptors **7–11** with the donor **5** were so efficient that the reactions virtually completed in 1 h at –78 °C to afford only β-mannosides in high yields (entries 1–5 in Table 1). The highly β-selective mannosylation was also achieved in the reaction of **5** with secondary alcohols **12–14** and with hindered tertiary alcohol **15** (entries 6–9). These results indicate that the present CB glycoside method for the β-mannopyranosylation is comparable to the Crich-Kahne sulfoxide method in terms of the yield and the stereoselectivity. For comparison, the glycosylation results by the sulfoxide method were also listed in Table 1 (parenthesis c of entries 2, 6, and 8) (*14d*). Unlike the sulfoxide method for β-mannopyranosylation (*14b*), toluene was also found to be a good solvent in the present method (parenthesis b of entry 1). The presence of the cyclic 4,6-acetal in mannosyl donors has been recognized as one of the necessary factors for the improvement of the β-selectivity in mannosylation: Crich et al. have suggested that the 4,6-benzylidene group stabilizes the intermediate α-triflate to give selectively the β-mannopyranosides via the S_N2-like displacement (*14a*). The present CB glycoside protocol could be applied to the glucosylation as well as the mannosylation. Thus, glucosylation of acceptors **7** and **9** with CB glucoside **6** provided α-glucosides as the major products (entries 11 and 12).

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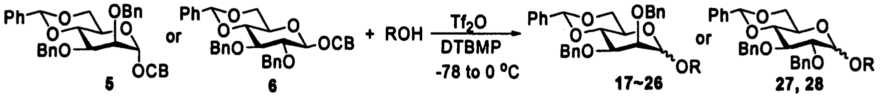
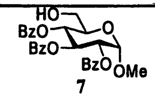
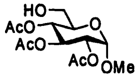
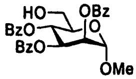
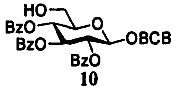
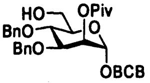
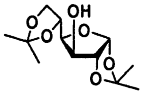
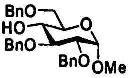
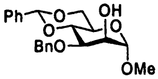
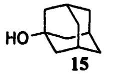
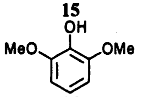
Table 1. Glycosylation with CB Glycosides 5 and 6



Entry	*Glycosyl Donor*	*Glycosyl Acceptor (ROH)*	*Product*	*Yield (%)*[a]	*Ratio (β/α)*[a]
1	5	 7	17	91 (95)[b]	β only (20:1)[b]
2	5	 8	18	95 [95][c]	β only [>25:1][c]
3	5	 9	19	91	β only
4	5	 10	20	85	β only
5	5	 11	21	83	β only
6	5	 12	22	91 [99][c]	>20:1[d] [19:1][c]
7	5	 13	23	93	16:1
8	5	 14	24	96 [96][c]	17:1 [15:1][c]
9	5	 15	25	90	>20:1[d]
10	5	 16	26	89	4:1
11	6	7	27	87	α only
12	6	9	28	85	α only

[a]Determined after isolation. [b]In toluene as a solvent. [c]The result by the Crich-Kahne sulfoxide method, see ref 14d. [d]After isolation of most of β-anomer, the ratio of the remaining β/α mixture was determined by ^1H NMR.

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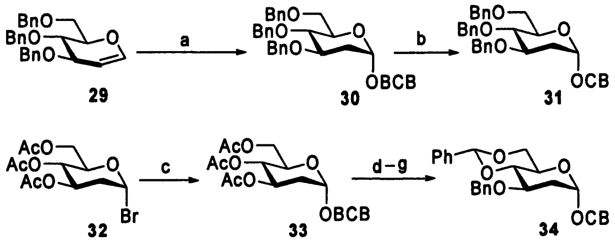
2. Direct construction of 2-deoxyglycosyl linkages

The stereocontrolled construction of 2-deoxyglycosyl linkages is more challenging than that of usual glycosyl linkages since there is no stereodirecting substituent at C-2 in 2-deoxyglycosyl donors and the resulting glycosides are more acid-labile. Although the synthesis of 2-deoxy-α-glycopyranosides is relatively easier to undertake due to the anomeric effect, the construction of 2-deoxy-β-glycopyranosyl linkages is far more challenging issue (*16*). Therefore, for the synthesis of 2-deoxy-β-glycopyranosides, the glycosyl donors with C-2 equatorial heteroatom substituents, which can participate during glycosylation to direct the incoming acceptor to the β-face and are removed at the later stage, were frequently used for the indirect methods (*17*). Direct methods using 2-deoxyglycopyranosyl donor for the construction of the 2-deoxy-β-glycopyranosyl linkage, however, would be far more valuable with respect to the synthetic efficiency and practicality. Although a few methods such as silver silicate (*18*) or silver zeolite (*19*) mediated glycosylation, the 2-deoxyglycosyl phosphite-TMSOTf method (*20*), and the glycosylation of 2-deoxyglycosyl fluoride with TiF_4 (*21*) or $SnCl_2$ (*22*), have been devised, the direct synthesis of 2-deoxy-β-glycopyranosides from 2-deoxyglycosyl donors still remains a difficult task. Thus, the CB glycoside methodology was applied to the direct synthesis of 2-deoxyglycopyranosides.

Scheme 2[a]



[a](a) **1**, $Ph_3P{\cdot}HBr$ (0.05 equiv), 0 to 40 °C, 1 h, 89%; (b) H_2, Pd/C, NH_4OAc, MeOH, 1 h, 90%; (c) **1**, $Hg(CN)_2$, $HgBr_2$, CH_3CN, 4Å MS, 0 °C, 20 min, 90%; (d) NaOMe, MeOH, 20 min; (e) $PhCH(OMe)_2$, CSA, DMF, 50 °C, 1 h, 90% over two steps; (f) BnBr, NaH, DMF, 1 h, 84%; (g) same as b), 92%.

CB tri-*O*-benzyl-2-deoxyglucoside **31** was readily obtained from tri-*O*-benzylglucal **29**, while CB 4,6-*O*-benzylidene-2-deoxyglucoside **34** was prepared from 2-deoxyglucosyl bromide **32** (*23*) as shown in Scheme 2. Glycosylations with these two CB 2-deoxyglycosyl donors **31** and **34** were performed by dropwise addition of Tf_2O to a stirred solution of the donor **31** or

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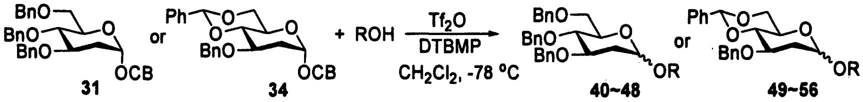
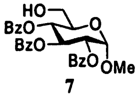
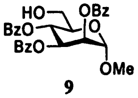
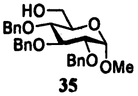
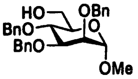
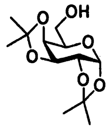
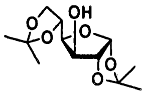
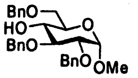
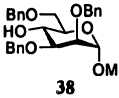
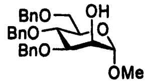
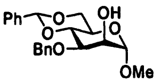
Table 2. Glycosylation of CB 2-deoxyglycosides 31 and 34


BnO
BnO
BnO
O
OCB
31
or
Ph
O
O
O
BnO
OCB
34
+ ROH
Tf2O
DTBMP
CH2Cl2, -78 °C
BnO
BnO
BnO
O
OR
40~48
or
Ph
O
O
O
BnO
OR
49~56

Entry	*Donor*	*Acceptor (ROH)*	*Product Yield (α/β)*	*Entry*	*Donor*	*Acceptor (ROH)*	*Product Yield (α/β)*
1	31	 7	40 98% (1:1)	10	34	7	49 92% (1:1)
2	31	 9	41 93% (1:1)	11	34	9	50 80% (1:1.2)
3	31	 35	42 94% (1:1.2)	12	34	36	51 83% (1:2)
4	31	 36	43 78% (1:1.5)	13	34	37	52 85% (1:5.1)
5	31	 37	44 93% (1:1.7)	14	34	12	53 76% (1:10)
6	31	 12	45 91% (α only)	15	34	13	54 72% (β only)
7	31	 13	46 91% (α only)	16	34	38	55 78% (1:10)
8	31	 38	47 88% (α only)	17	34	 39	56 71% (1:8)
9	31	 14	48 94% (9.4:1)				

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34, the acceptor, and DTBMP in the presence of 4Å molecular sieves in CH_2Cl_2 at −78 °C. Glycosylation of primary alcohol acceptors **7**, **9**, **35**–**37** with the benzyl-protected donor **31** exhibited no significant stereoselectivity (entries 1–5 in Table 2). Only the slight β-selectivity was observed when primary alcohols **35** (α/β=1:1.2), **36** (α/β=1:1.5), and **37** (α/β=1:1.7) were employed as the glycosyl acceptors (entries 3–5). On the other hand, glycosylation of secondary alcohol acceptors **12**, **13**, **38** with **31** afforded exclusively α-disaccharides (entries 6–8) and of secondary alcohol **14** furnished predominantly (α/β=9.4:1) α-disaccharide **48** (entry 9).

Under the same reaction condition, glycosylation of primary alcohol acceptors **7** and **9** with the benzylidene-protected donor **34** provided a mixture of almost equal amount of α- and β-anomers of disaccharides **49** and **50**, respectively (entries 10 and 11). When other primary alcohols **36** and **37** were used as glycosyl acceptors, the ratio of the β-anomer of the resulting disaccharides were increased substantially indicating α/β=1:2 for **51** and 1:5.1 for **52**, respectively (entries 12 and 13). The highly β-selective glycosylation could be achieved by employing the donor **34** with secondary alcohol acceptors. Thus, the α:β ratio in the reactions of **34** with both acceptors **12** and **38** was 1:10 and that with the acceptor **39** was 1:8 (entries 14, 16, and 17). Interestingly, the coupling of **34** with the acceptor **13**, on the other hand, afforded exclusively the β-anomer of disaccharide **54** in 72% yield (entry 15).

Complete reversal of the stereoselectivity, from α to β, simply by changing the benzyl-protective group to the benzylidene-protective group of the 2-deoxyglycosyl donor in the present glycosylation is unprecedented, although the precise mechanistic details underlying this reversal of selectivity and the lack of selectivity with primary alcohol acceptors are not yet clear.

3. Acceptor-dependent stereoselective β-D-arabinofuranosylation

Recently the development of an efficient and sterocontrolled β-arabinofuranosylation is getting more important due to the presence of β-oligoarabinofuranosides in arabinogalactan and immunogenic lipoarabinomannan found in mycobacterial cell walls. The synthesis of these oligoarabinofuranosides could greatly contribute to the development of new therapeutic agents against tuberculosis and other mycobacterial infections (*24*). To date the reliable direct methods for the construction of β-arabinofuranosyl linkages have not yet been established, although a few indirect methods have been reported for the synthesis of β-D-arabinofuranosides (*25*). Thioarabinofuranosides have been used as glycosyl donors for the direct synthesis of pentaarabinofuranosides and hexaarabinofuranosides containing β-D-arabinofuranosyl linkages (*26*), but they are not generally applicable for β-

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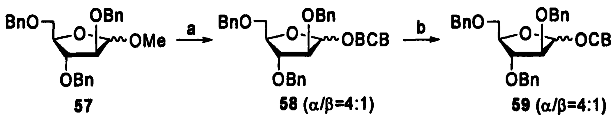
arabinofuranosylation with a range of glycosyl acceptors (β/α≤4.5:1) (*27*). Surprisingly, the CB glycoside method for the direct construction of the β-arabinofuranosyl linkage, on the other hand, was turned out to be a generally applicable and highly stereoselective method, in which the stereoselectivity was achieved by properly choosing protective groups on glycosyl acceptors.

CB tribenzyl-D-arabinofuranoside **59** was efficiently prepared from methyl tribenzyl-D-arabinofuranoside **57** as shown in Scheme 3. Treatment of **57** with acetyl bromide in trifluoroacetic acid and subsequent coupling of the resulting crude arabinofuranosyl bromide with benzyl 2-(hydroxymethyl)benzoate (**1**) followed by selective hydrogenolysis of the resulting BCB arabinofuranoside **58** (α/β=4:1) afforded the desired CB arabinoside **59**. CB 3,5-di-*O*-benzoyl-2-*O*-benzylarabinofuranoside **60** was also prepared in like fashion.

Scheme 3[a]



[a](a) (i) CH_3COBr, TFA, CH_2Cl_2, rt, 2 h; (ii) **1**, $HgBr_2$, $Hg(CN)_2$, CH_3CN, rt, 30 min, 83% in two steps; (b) H_2, Pd/C, NH_4OAc, MeOH-EtOAc (1:1), rt, 1 h, 89%.

Glycosylations of various acceptors with arabinofuranosyl donors **59** and **60** were carried out by dropwise addition of Tf_2O to a stirred solution of the donor **59** or **60**, the acceptor, and DTBMP in the presence of 4Å molecular sieves in CH_2Cl_2 at −78 °C. The result of the glycosylation was unprecedented and exciting in terms of the stereochemistry of products. Thus, reaction of the donor **59** with acceptor **7** having benzoyl-protective groups afforded β-disaccharide **66** almost exclusively (β/α=99:1) in 97% yield (entry 1 in Table 3), while the same reaction with acceptor **35** having benzyl-protective groups gave a mixture of α- and β-disaccharides **71** (β/α=7:1) (entry 6). Further examples clearly showed that the protective group of glycosyl acceptors was the crucial factor for the outcome of the stereochemistry in glycosylations with **59**. Regardless of pyranoses or furanoses and of primary alcohols or secondary alcohols, glycosylations of acceptors having benzoyl-protective groups, **9**, **61**–**63** with the donor **59** afforded β-disaccharides either exclusively or predominantly (β/α≥20:1) in high yields (entries 2–5), whereas the β-stereoselectivity in glycosylations of acceptors having benzyl-protective groups, **36**, **38**, **64**, **65** with the donor **59** was much less pronounced (β/α≤11:1) (entries 7–10). The influence of other protective groups of the glycosyl acceptor on the stereochemistry of the glycosylation product was also observed. Thus, the glycosylations of acceptor **8** having acetyl groups and of acceptor **14** having a

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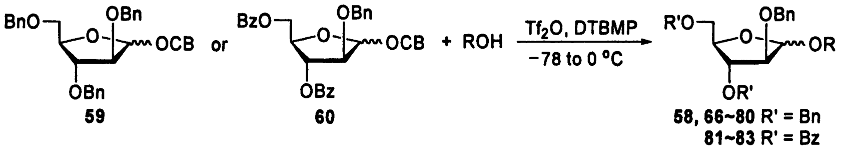
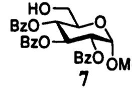
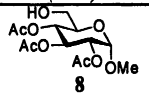
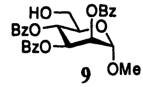
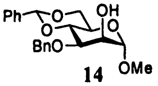
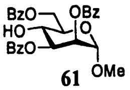
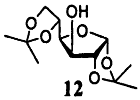
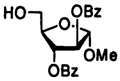
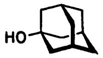
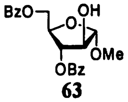
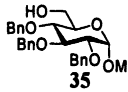
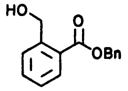
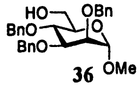
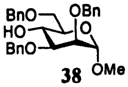
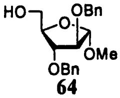
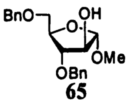
Table 3. Glycosylation with CB arabinofuranosides 59 and 60


BnO
OBn
O
OCB
OBn
59
or
BzO
OBn
O
OCB
OBz
60
+ ROH
Tf2O, DTBMP
-78 to 0 °C
R'O
OBn
O
OR
OR'
58, 66~80 R' = Bn
81~83 R' = Bz

Entry	Donor	Acceptor (ROH)	Product Yield[a] (β/α)[b]	Entry	Donor	Acceptor (ROH)	Product Yield[a] (β/α)[b]
1	59	 7	66 97% (99:1)	11	59	 8	76 88% (38:1)
2	59	 9	67 95% (β only)	12	59	 14	77 92% (40:1)
3	59	 61	68 86% (20:1)	13	59	 12	78 89% (6.1:1)
4	59	 62	69 92% (β only)	14	59	 15	79 81% (β only)
5	59	 63	70 91% (21:1)	15	59	1-octanol	80 88% (14:1)
6	59	 35	71 95% (7:1)[c]	16	59	 1	58 81% (4:1)
7	59	 36	72 95% (4:1)[c]	17	60	9	81 75% (13:1)
8	59	 38	73 89% (11:1)	18	60	35	82 71% (2:1)[c]
9	59	 64	74 92% (6.7:1)	19	60	15	83 65% (7:1)[c]
10	59	 65	75 90% (2.2:1)				

[a]Determined after isolation. [b]Determined by HPLC using Nova-Pak® C18 column. [c]Determined by ^1H NMR.

benzylidene group with the donor **59** afforded predominantly β-disaccharides **76** (β/α=38:1) and **77** (β/α=40:1), respectively (entries 11 and 12). On the other hand, the glycosylation of acceptor **12** having two isopropylidene groups with the donor **59** afforded a mixture of α- and β-disaccharides **78** (β/α=6.1:1) (entry 13). Glycosylation of hindered tertiary alcohol **15** with **59** afforded only β-arabinofuranoside **79** (entry 14), whereas glycosylations of primary alcohols, 1-octanol and **1** with **59** gave mixtures of α- and β-arabinofuranosides (entries 15 and 16). Glycosylations with the CB dibenzoylarabinofuranoside **60** as a donor, however, were not as stereoselective as with **59** (entries 17–19). These results indicate that glycosylation employing CB tri-*O*-benzylarabinoside **59** as glycosyl donor is a reliable and generally applicable direct method for the construction of β-arabinofuranosyl linkages and the acyl-protective groups on glycosyl acceptors are essential for the β-stereoselectivity.

4. Application to oligosaccharide synthesis

The CB glycoside methodology has been applied to the synthesis of some oligosaccharides, for example, protected trisaccharide **89**, which is the repeat unit of the atypical O-antigen polysaccharide from Danish *Helicobacter pylori* strains (*12a*), protected tetrasaccharide **98**, which was designed as an analog of the tetrasaccharide repeat unit of the O-antigen polysaccharide from the *E. coli* lipopolysaccharide (*12b*), and octaarabinofuranoside **112** in arabinogalactan and lipoarabinomannan found in mycobacterial cell wall (*11*). For the rapid assembly of oligosaccharides, the latent-active glycosylation method is a valuable strategy, which has certain advantage over the other strategies since it does not require the tuning of the anomeric leaving group so that the glycosyl donor and the acceptor could be prepared from a common building block and the sequential glycosylation could be performed employing a single glycosylation method. The following CB-mediated synthesis of three oligosaccharides, namely **89**, **98**, and **112**, would show that the CB glycoside methodology employing the pair of the latent BCB glycoside and the active CB glycoside is a reliable general method for the synthesis of various complex oligosaccharides.

Coupling of donor CB L-rhamnoside **84** and acceptor BCB D-rhamnoside **85** by addition of Tf_2O to the mixture of **84**, **85**, and DTBMP gave desired α-disaccharide **87** in 88% yield (Scheme 4). Selective hydrogenolysis of the BCB disaccharide **87** afforded CB disaccharide **88** in 95% yield. Under the same reaction condition, the glycosylation of BCB 3-*C*-methylmannoside acceptor **86** with the CB disaccharide donor **88** afforded the target α-trisaccharide **89** along with its β-anomer in 7:1 ratio in 80% yield. This result indicates that the participating group at C-2 is working well in the CB glycosides.

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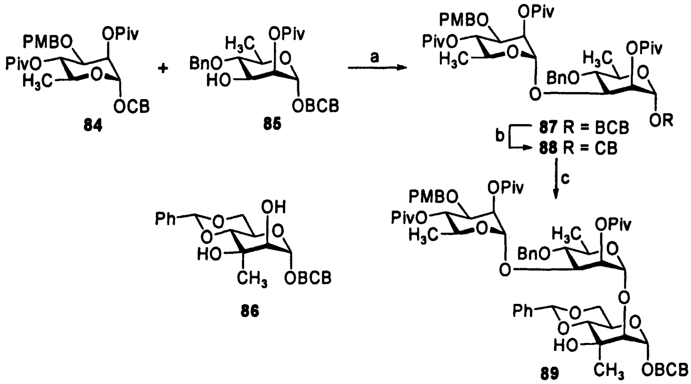
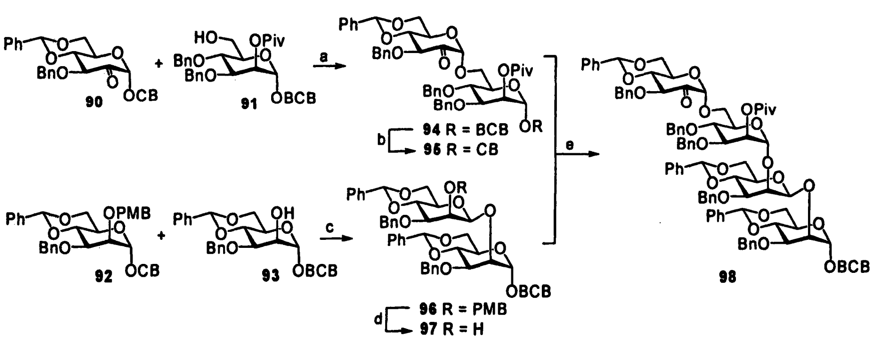
Scheme 4[a]


PMBO
OPiv
PivO
H_3C
OCB
84
+
BnO
HO
OBCB
85
a
OR
b
87 R = BCB
88 R = CB
c
Ph
OH
CH_3
86
89

[a](a) DTBMP, 4Å MS, CH_2Cl_2, rt, 10 min, then Tf_2O, –78 to 0 °C, 2 h, 88%; (b) H_2, Pd/C, NH_4OAc, MeOH, rt, 1 h, 95%; (c) **86**, DTBMP, 4Å MS, CH_2Cl_2, rt, 10 min, then Tf_2O, –78 to 0 °C, 2 h, 80% (α:β=7:1).

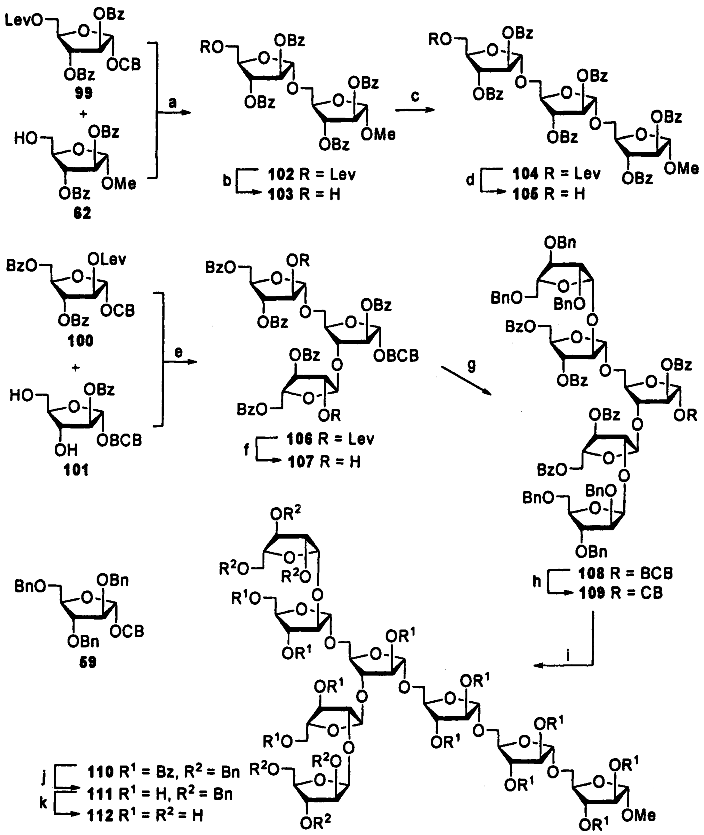
Coupling of the ketone glycosyl donor **90** and acceptor **91** was conducted by addition of Tf_2O to the mixture of **90**, **91**, and DTBMP to provide a mixture of α-disaccharide **94** and its anomeric β-disaccharide (4:1) in 74% yield (Scheme 5). Glycosylation of acceptor **93** with glycosyl donor **92**, on the other hand, carried out under the different reaction condition for the synthesis of β-mannoside **96**. Thus, the donor **92** was first activated by the addition of Tf_2O and then reacted with the acceptor **93** to afford exclusively the β-disaccharide **96** in

Scheme 5[a]


Ph
BnO
90
OCB
+
HO
OPiv
91
OBCB
a
b
94 R = BCB
95 R = CB
OR
OPMB
92
OH
93
c
96 R = PMB
97 R = H
d
e
98

[a](a) **90**, **91**, DTBMP, 4Å MS, CH_2Cl_2, –40 °C, then Tf_2O, –40 to 0 °C, 3 h, 74% (α/β=4:1); (b) H_2, Pd/C, NH_4OAc, MeOH, rt, 1 h, 87%; (c) **92**, DTBMP, Tf_2O, 4Å MS, CH_2Cl_2, –78 °C, 10 min, then **93**, –78 to 0 °C, 3 h, 91%; (d) DDQ, CH_2Cl_2-H_2O (18:1), rt, 9 h, 83%; (e) **95**, **97**, DTBMP, 4Å MS, CH_2Cl_2, –40 °C, then Tf_2O, –40 to 0 °C, 3 h, 75%.

Scheme 6[a]


99
62
a
b
102 R = Lev
103 R = H
c
d
104 R = Lev
105 R = H
100
101
e
106 R = Lev
107 R = H
f
g
108 R = BCB
109 R = CB
h
59
i
j
k
110 R¹ = Bz, R² = Bn
111 R¹ = H, R² = Bn
112 R¹ = R² = H

[a](a) DTBMP (3.0 equiv), 4Å MS, CH_2Cl_2, then Tf_2O (1.3 equiv), –40 to 0 °C, 2 h, 84%; (b) NH_2NH_2(aq)-AcOH (1:2), THF-MeOH (10:1), rt, 1 h, 97%; (c) **99** (1.7 equiv), DTBMP (4.7 equiv), 4Å MS, CH_2Cl_2, then Tf_2O (2.3 equiv), –40 to 0 °C, 2 h, 75%; (d) same as (b), 90%; (e) DTBMP (1.6 equiv), 4Å MS, CH_2Cl_2, then Tf_2O (1.2 equiv), –40 to 0 °C, 2 h, 84%; (f) same as (b), 99%; (g) **59** (3.7 equiv), DTBMP (11.0 equiv), 4Å MS, CH_2Cl_2, then Tf_2O (5.5 equiv), –78 to 0 °C, 2 h, 82%; (h) H_2, $Pd(OH)_2$, NH_4OAc (1.1 equiv), MeOH-EtOAc (1:1), rt, 1 h, 83%; (i) **105** (1.5 equiv), DTBMP (3.0 equiv), 4Å MS, CH_2Cl_2, then Tf_2O (1.5 equiv), –78 to 0 °C, 2 h, 83%; (j) NaOMe (16.7 equiv), MeOH, CH_2Cl_2, rt, 3 h, 97%; (k) H_2, Pd/C, AcOH-H_2O (4:1), rt, 3 h, 91%.

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91% yield and removal of the PMB group in **96** with DDQ gave alcohol **97**. It has been demonstrated that CB mannopyranosides having the 4,6-*O*-benzylidene group and a C-2 non-participating group are extremely useful mannosyl donors for the stereoselective β-mannopyranosylation. The latent BCB disaccharide **94** was converted into the active CB disaccharide **95** by the selective hydrogenolysis. Finally, the coupling of two disaccharides **95** and **97** was performed by addition of Tf_2O to a stirred solution of the glycosyl donor **95** and the acceptor **97** to afford exclusively the desired α-tetrasaccharide **98** in 75% yield.

For the synthesis of octaarabinofuranoside **112**, the acceptor-dependent β-arabinosylation method was applied as shown in Scheme 6. Reaction of CB arabinofuranosyl donor **99** and acceptor **62** afforded α-arabinofuranosyl disaccharide **102** in 84% yield and subsequent removal of the levulinyl group in **102** with hydrazine gave alcohol **103**. Repetitive glycosylation of the disaccharide **103** as an acceptor with the arabinofuranosyl donor **99** gave α-trisaccharide **104**, which was converted into trisaccharide acceptor **105** by removal of its levulinyl group with hydrazine. Glycosylation of diol **101** with glycosyl donor **100** afforded α-trisaccharide **106** in 84% yield without any problems. Deprotection of the two levulinyl groups in **106** with hydrazine gave diol **107**, in which the benzoyl group was utilized as the protective group for acceptor-dependent β-arabinofuranosylation in the next step. The crucial double β-arabinofuranosylation of the diol **107** with the arabinofuranosyl donor **59** proceeded smoothly to afford pentaarabinofuranoside **108** in 82% yield with complete β-selectivity. No α-glycosides were detected at all in the reaction mixture. The latent BCB arabinofuranoside **108** was converted into the active CB arabinoside **109**. Coupling of the pentaarabinofuranosyl donor **109** and the triarabinofuranosyl acceptor **105** afforded protected octaarabinofuranoside **110** in 83% yield. Debenzoylation of **110** with sodium methoxide followed by hydrogenolysis of the resulting partially benzyl-protected octaarabinfuranoside **111** afforded the desired octaarabinofuranoside **112**.

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5. Conclusion

We have described the synthesis of novel CB glycosides and highly efficient and steroselective procedures for the β-mannopyranosylation, 2-deoxyglycosylation, and β-D-arabinofuranosylation employing CB glycosides as glycosyl donor with triflic anhydride. The CB glycoside method was found to be comparable to the thioglycoside and the glycosyl sulfoxide methods in terms of stereoselectivity for β-mannopyranosylation and a highly α- and β-stereoselective (dual stereoselective) method by using differently protected CB 2-deoxyglycosides as glycosyl donor for 2-deoxyglycosylation. And, the CB glycoside method was also found to be a reliable and generally applicable direct method for the stereoselective β-arabinofuranosylation employing a CB-tri-*O*-benzylarabinoside as glycosyl donor and the glycosyl acceptors with the acyl-protective group.

In addition, the CB glycoside method has been successfully applied to the synthesis of some important oligosaccharides, trisaccharide **89**, tetrasaccharide **98**, and octaarabinofuranoside **112** as the latent-active glycosylation method, which is a valuable strategy for the rapid assembly of oligosaccharides.

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Chapter 10

Oligosaccharide Synthesis with Glycosyl Phosphates

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This chapter desribes the synthesis of glycosyl phosphates and their use as glycosylating agents. Their use in the construction of complex oligosaccharides, both in solution and on solid support, is presented. Their ease of synthesis in combination with their rapid activation have made them ideal glycosylating agents to be used for automated solid phase oligosaccharide synthesis.

Introduction

The preparation of synthetic oligosaccharide sequences presents a significant challenge to the organic chemist.*(1)* The desired structures possess a myriad of hydroxyl groups, are often highly branched and require the stereospecific formation of glycosidic linkages. Nature employs glycosyltransferases to catalyze the formation of a new glycosidic linkage. These enzymes utilize nucleotide diphosphosugars (NDPs) as substrates.*(2)* The preparation of NDPs is most commonly accomplished by the coupling of glycosyl 1-phosphates and nucleoside 5'-monophosphates. The variety of procedures available for the synthesis of anomeric phosphates was in stark contrast to the limited uses of glycosyl phosphates as glycosylating agents. Recent advancements, however, have resulted in an increased interest in phosphorus based glycosyl donors *(3)* that was initiated by Ikegami and co-workers.*(4)*

In Frontiers in Modern Carbohydrate Chemistry; Demchenko, A.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2007.

Here we discuss the chemical synthesis of glycosyl 1-phosphates and their application in oligosaccharide construction. The focus of this review is on chemical methods and not enzymatic approaches, which have been the subject of excellent reviews.*(5)* The synthesis of anomeric phosphates from glycal starting materials and the utilization of glycosyl phosphate triesters in the construction of *O*-glycosides is discussed. Finally, solid phase techniques employing glycosyl phosphates for the automated synthesis of complex structures are presented.

Synthesis of Glycosyl Phosphate Triesters

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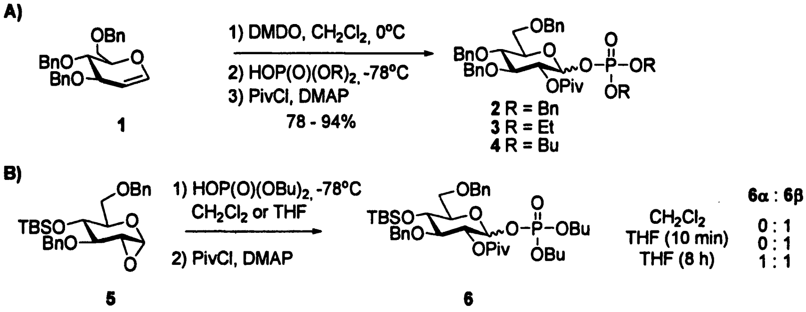
Generation of anomeric phosphate triesters for use as glycosylating agents and for the synthesis of NDPs has been accomplished from a variety of intermediates.*(3)* Anomeric lactols and 1,2-anhydro sugars are commonly employed, while derivitization of other glycosylating agents (glycosyl imidates, *n*-pentenyl-, bromo- and thioglycosides), has also seen use in generating glycosyl phosphates. These methods allow for the construction of phosphate triesters containing various alkyl and aryl substituents.

Glycals are attractive starting materials for the synthesis of glycosylating agents since they possess only three hydroxyl groups that need to be differentiated. Furthermore glycal derived 1,2-anhydrosugars can be transformed into fully protected glycosyl phosphates in a one-pot procedure.*(6, 7)* Thus, generation of the anhydrosugar with dimethyldioxirane followed by epoxide opening at low temperature and *in situ* acylation afforded building blocks **2**–**4** (Scheme 1A). This method worked well for both monosaccharide and disaccharide glycal moieties. Alpha or beta-enriched phosphates can be generated with this method depending on the solvent employed for the 1,2-anhydrosugar opening. Coordinating solvents such as THF afforded predominantly α-phosphates while less polar solvents such as dichloromethane resulted in β-selective epoxide opening. The latter can be rationalized by the finding that anomerization takes place more rapidly in THF (Scheme 1B).*(7b)* For example, when the ring-opening of 1,2-anhydrosugar **5** was carried out in CH_2Cl_2 followed by acylation, β-phosphate **6** was obtained exclusively. Similarly, when the ring-opening was performed in THF and the newly generated C2-OH was immediately acylated, only the β-phosphate was obtained. When the ring-opening was allowed to stir at ambient temperature for 8 h and then acylated, a 1:1 mixture of anomers **6a** and **6b** was obtained. These results support earlier findings that α-phosphates could be formed from the β-isomers by acid-catalyzed anomerization. The possibility to create different types of anomeric phosphates proved particularly important with respect to the glycosylation properties of the ensuing species, since (as with most types of glycosyl donors) the β-phosphates are a lot more reactive than their α-counterparts.*(7b)*

The installation of C2 protecting groups other than esters proved challenging.[7,8] Benzylation employing sodium hydride and benzyl bromide resulted in migration of the phosphate to yield the C2-phosphoryl benzyl glycoside. Triethylsilyl ethers, on the other hand, were readily prepared by reaction of the C2-hydroxyl group of the glycosyl phosphate with triethylsilyl chloride and imidazole in DMF. In pure form, α- and β-glycosyl phosphates were found to be completely stable to storage for several months at 0°C.



Scheme 1. Construction of glycosyl phosphates from glycals.

Construction of Glycosidic Bonds Using Glycosyl Phosphates

Glycosyl phosphates have now been applied for the assembly of a variety of both *C*- and *O*-glycosides.*(3, 9)* A representative collection of *O*-glycosylations employing alkyl- and aryl phosphates is summarized in Table 1. As can be seen, several different glycosyl phosphates have been used, ranging from reactive (*e.g.* fucosyl phosphate **7**, entry 1) to relatively unreactive (*e.g.* glucuronic acid phosphate **24**, entry 7) glycosylating agents. A stoichiometric amount of trimethylsilyl trifluoromethanesulfonate (TMSOTf) has been shown to be the promoter of choice to effect complete activation of glycosyl phosphates (other Brønsted and Lewis acids, such as $SnCl_2$, ZnI_2, $Zn(OTf)_2$ gave moderate results).*(4, 7b)* A whole range of protecting groups has been shown to be compatible with the Lewis-acidic conditions used to activate glycosyl phosphates, including acetals and the tetraisopropyldisilyloxanylidene group (compounds **31** and **32**, entry 9). However, the triethylsilyl group on the C2-OH

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of a glucosyl phosphate (**10**, entry 2) was lost during the condensation with galactose **11**. It should be noted that several aryl and alkyl phosphotriesters can be used as glycosyl donors and that there seems to be little difference in reactivity between them, although this has not been scrupulously investigated.

To streamline oligosaccharide assembly and circumvent intermediate protecting group manipulations, glycosyl phosphates were used in a series of regioselective, chemoselective and orthogonal condensation reactions. For example, glucoside **36** was used to glycosylate diacetone galactose **11** to give disaccharide **37**, which in turn was employed in the next condensation event to provide trimer **39** (Scheme 2).*(7b)* Notably, this sequence of reactions can also be executed in a one-pot fashion. Other regioselective condensations included glycosidations of diacetonegalactose **11** using C3-OH galactosylphosphate **40**, C2-OH glucuronic acid phosphate **41**, and C2-OH glucosyl phosphate **42**.

As outlined above, α- or β-anomeric glycosyl phosphates can both be synthezised in good yield. In general, α-configured phosphates are more stable than their β-linked counterparts and require higher temperatures to effect glycosylation: the more reactive β-glycosyl phosphates in the glucose and galactose series were activated at -78°C while their α-anomeric counterparts were inert under those conditions and required higher temperatures (-40°C → -20°C) for activation. This intrinsic difference in reactivity opened up the way to explore the selective activation of the more reactive over the least reactive phosphate, thereby achieving a chemoselective glycosylation protocol (Scheme 3).*(7b)* To this end, a mixture of α-galactosyl phosphate **43** and β-glucosyl phosphate **38** was treated with a stoichiometric amount of TMSOTf at low temperature to result in the selective formation of the disaccharide phosphate **44**. In the ensuing condensation step the activation of the α-phosphate was achieved at higher temperature to glycosylate diacetone galactose **11** to give trimer **45**.

Thioglycosides have been extensively used in both chemoselective and orthogonal glycosylation strategies. Since thioglycosides are completely stable to the activation conditions used in glycosyl phosphate couplings, an orthogonal strategy where thioglycoside **46** functioned as the central building block was explored (Scheme 4).*(7a)* Activation of **38** with TMSOTf at -78°C in the presence of **46** furnished disaccharide **47** in good yield. Coupling of **47** with glycal acceptor **48** was then initiated by MeOTf/DTBP activation to afford trisaccharide **49**.

Next to glycosyl phosphates, a variety of glycosyl phosphate analogues have been synthesized and evaluated as glycosylating agents. These analogs include glycosyl phosphorimidates, phosphoramidates, phosphoramidimidates, thiophosphates, phosphorodithioates and phosphoroselenoates. The use these analogs has recently been reviewed.*(14)*

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Table 1. *O*-glycosylations using glycosyl phosphates.

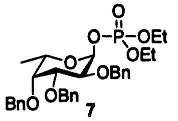
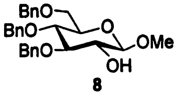
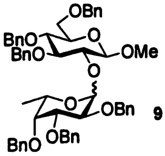
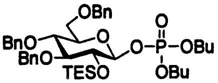
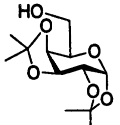
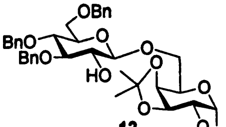
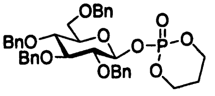
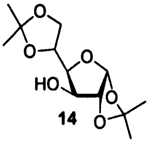
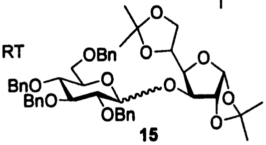
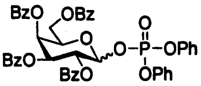
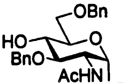
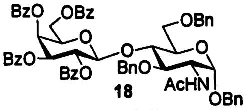
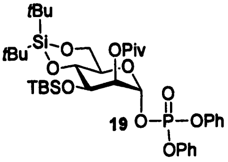
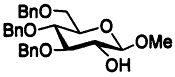
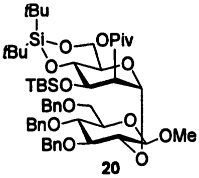
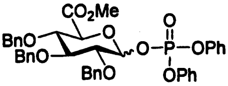
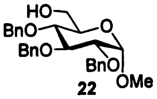
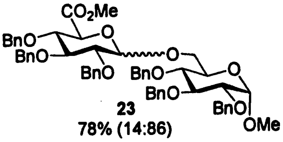
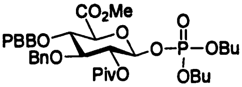
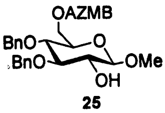
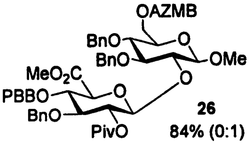
Entry	Donor	Acceptor	Solvent/ Temperature/ Reaction time	Product Yield (α/β)	Ref
1	 **7**	 **8**	CH_2Cl_2 -20°C 10 min	 **9** 95% (3:2)	7b
2	 **10**	 **11**	CH_2Cl_2 -78°C 30 min	 **12** 71% (0:1)	7a
3	 **13**	 **14**	CH_2Cl_2 -78°C to RT 25 min	 **15** 67% (1:12)	10
4	 **16**	 **17**	CH_2Cl_2 RT 1h	 **18** 83% (0:1)	4
5	 **19**	 **8**	CH_2Cl_2 -78°C 30 min	 **20** 75% (1:0)	7c
6	 **21**	 **22**	EtCN -78°C 5-10 min	 **23** 78% (14:86)	4
7	 **24**	 **25**	CH_2Cl_2 -20°C 30 min	 **26** 84% (0:1)	7b

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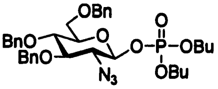
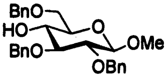
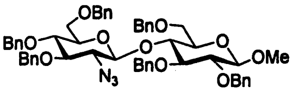
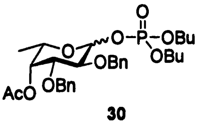
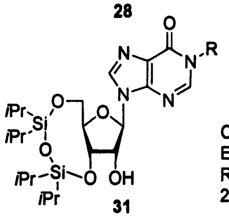
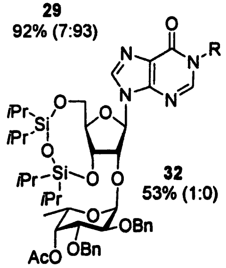
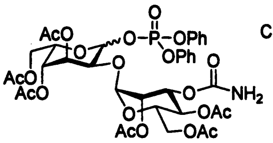
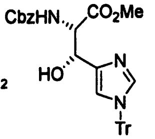
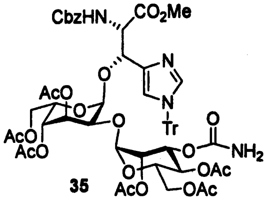
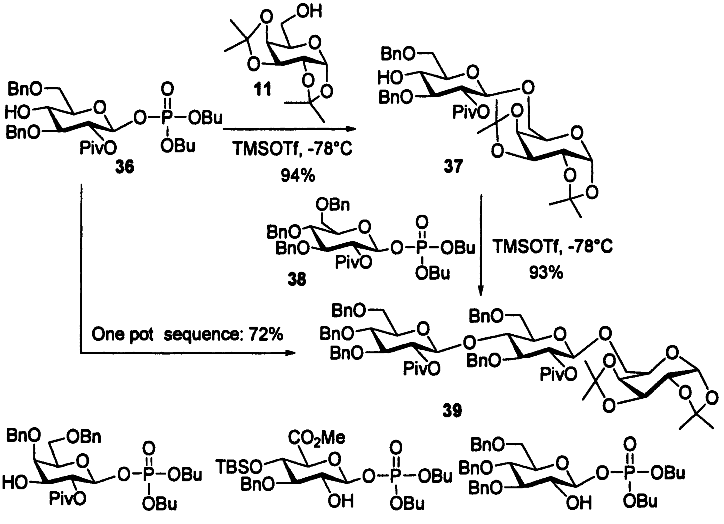
Table 1. *Continued.*

Entry	Donor	Acceptor	Solvent/ Temperature/ Reaction time	Product Yield (α/β)	Ref
8	 **27**	 **28**	EtCN -78°C 2h	 **29** 92% (7:93)	11
9	 **30**	 **31**	ClCH$_2$CH$_2$Cl/ Et$_2$O RT 20 min	 **32** 53% (1:0)	12
10	 **33**	 **34**	CH$_2$Cl$_2$ 0°C 20 min	 **35** 54-63%	13



11
36
TMSOTf, -78°C
94%
37
38
TMSOTf, -78°C
93%
One pot sequence: 72%
39
40
41
42

Scheme 2. Regioselective glycosylation using glycosyl phosphates.

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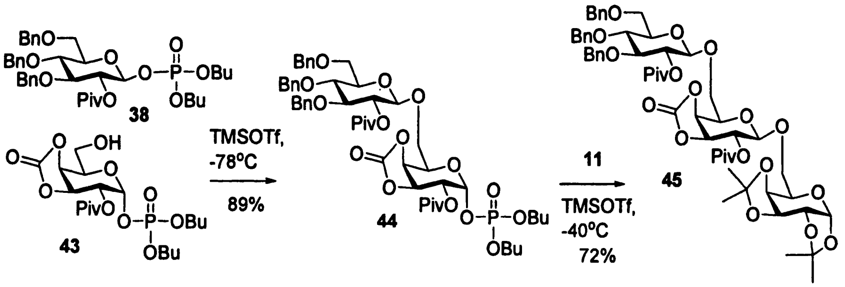
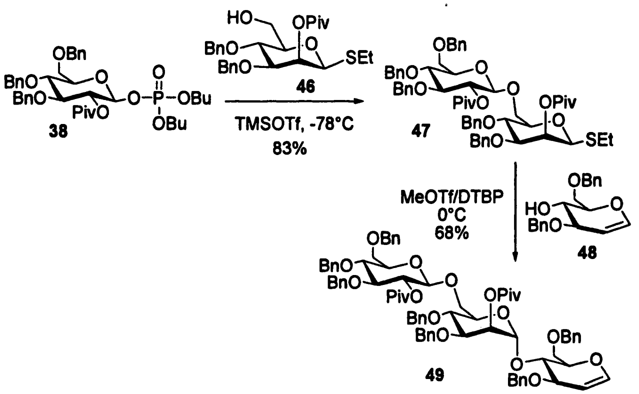

38
43
TMSOTf,
-78°C
89%
44
11
TMSOTf,
-40°C
72%
45

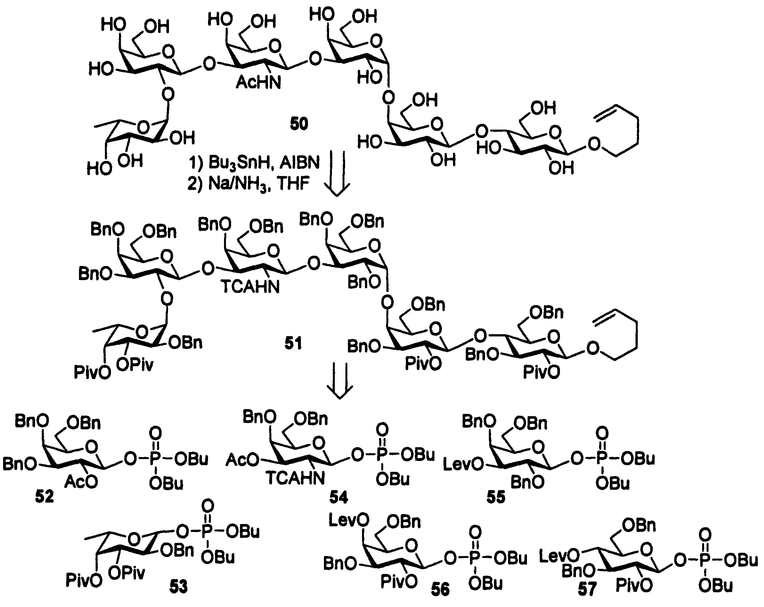
Scheme 3. Chemoselective glycosylation using glycosyl phosphates.


38
46
TMSOTf, -78°C
83%
47
MeOTf/DTBP
0°C
68%
48
49

Scheme 4. Orthogonal glycosylation strategy using glycosyl phosphates.

Synthesis of Complex Oligosaccharides Using Glycosyl Phosphates

Glycosyl phosphates have only recently been used for the assembly of larger oligosaccharides. We have explored several solution phase syntheses of complex oligosaccharides, using glycosyl phosphates, with the ultimate goal to develop the automated solid phase assembly of the target structures. In keeping with this goal, a linear assembly strategy using monomeric building blocks was followed. For example, the tumor-associated carbohydrate antigen Globo-H **50** was synthesized from the six glycosyl phosphate building blocks **52-57**, as depicted retrosynthetically in Scheme 5.*(15)* The galactosyl phosphate building block **55**, bearing a C2-*O*-benzyl ether, was generated from the corresponding galactosyl trichloroacetimidate, since it could not be synthesized using the standard one-pot procedure. The galactosamine phosphate building block **54** was procured using the same strategy. It is of interest to note that we found that *N*-acyl protected glucosamine and galactosamine phosphates perform slightly better than their trichloroacetimidate congeners. In the assembly of the Globo-H hexamer the crucial α-Gal-(1→4)-Gal linkage was succesfully installed using galactosyl phosphate **55**, at low temperature (-78°C) in a CH_2Cl_2/Et_2O-solvent mixture.



Scheme 5. Synthesis of the Globo-H hexasaccharide using glycosyl phosphates.

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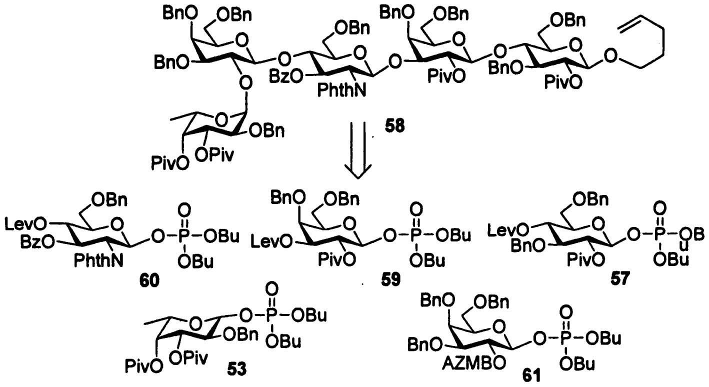
The fully protected H-type II pentasaccharide **58** (Scheme 6) was also synthesized in a linear fashion.[16] In this assembly, the applicability of the 2-(azidomethyl)benzoyl group (AZMB, selectively cleavable by reduction using phosphines *(17)*) in carbohydrate synthesis was explored. It turned out that this group performed well as a participating group in the construction of the pentasaccharide in combination with the building blocks shown in Scheme 6.*(18)* The target compound was constructed from the five monomers, thereby demonstrating the use of glycal-derived glycosyl phosphates in complex oligosaccharide synthesis and paving the way for a solid phase approach (*vide infra*).

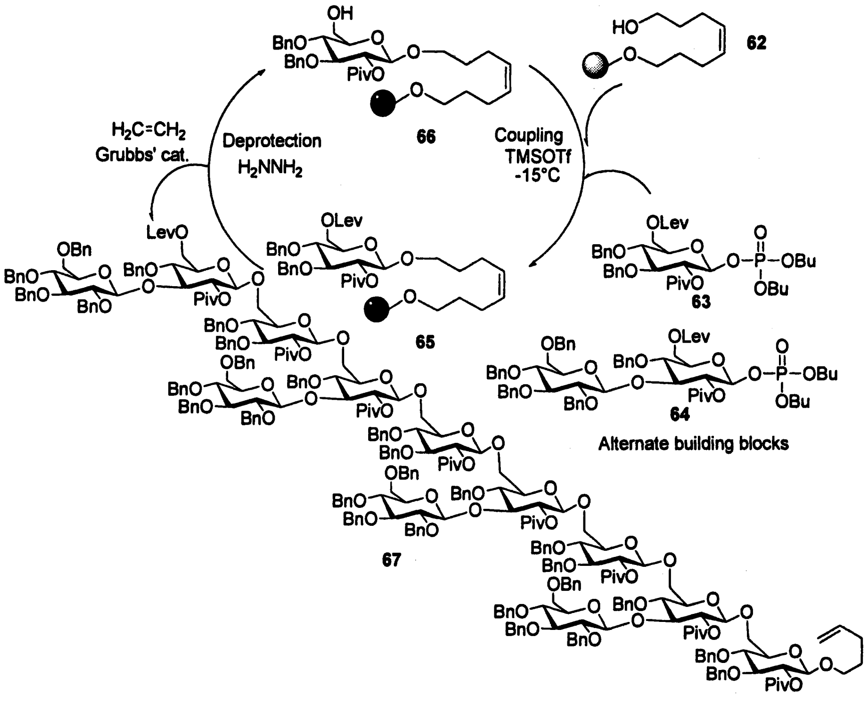
Use of Glycosyl Phosphates on Solid Support

Glycal derived glycosyl phosphates have proven to be readily accessible and effective glycosyl donors as detailed above. They require only liquid, non-toxic activators, and generally the addition of molecular sieves is unnecessary. Therefore, glycosyl phosphates lend themselves particularly well to the synthesis of oligosaccharides on solid support. Initially, we manually assembled a β-(1→6)-linked triglucoside and a more challenging β-(1→4)-linked triglucoside on a Merrifield resin, functionalized with an octenediol linker system (**62**, Scheme 7).*(19)* Following the success of glycosyl phosphates in the manual solid



Scheme 6. Synthesis of the H-type II pentasaccharide using glycosyl phosphates.

support synthesis of oligosaccharides, their application to the automated solid-phase synthesis of oligosaccharides was evaluated.*(20)* The fully protected phytoalexin elicitor (PE) β-glucan **67** was selected as a complex target structure as it had been synthesized before in solution *(21)* and on solid support *(22)* and would serve well as a benchmark for the automation of glycosyl phosphates (Scheme 7). For the synthesis of the branched β-(1→3)/β-(1→6) structure, two different glycosyl phosphate donors, **63** and **64**, were synthesized. Levulinoyl esters were employed as 6-*O* temporary protecting groups and the 2-*O*-pivaloyl group was used to ensure complete β-selectivity in the glycosylation reaction. Each cycle incorporated double glycosylations (at -15°C) and double deprotections to ensure high yielding steps (Table 2). A branched hexasaccharide was constructed in ten hours in over 80% yield as judged by HPLC analysis. Also, dodecasaccharide **67** was prepared in a fully automated fashion in 17 hours and in 50% yield using the same cycle.



Scheme 7. Automated solid phase synthesis of a protected phytoalexin elicitor.

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Table 2. General cycle used with glycosyl phosphates 63, 64 for the construction of 67.

Step	*Function*	*Reagent*	*Time (min)*
1	Couple	5 eq. building block and 5 eq. TMSOTf	30
2	Wash	Dichloromethane	6
3	Couple	5 eq. building block and 5 eq. TMSOTf	30
4	Wash	1:9 methanol:dichloromethane	4
5	Wash	Tetrahydrofuran	4
6	Wash	3:2 pyridine:acetic acid	3
7	Deprotection	2×20 eq. hydrazine (3:2 pyridine:acetic acid)	30
8	Wash	3:2 pyridine: acetic acid	3
9	Wash	1:9 methanol:dichloromethane	4
10	Wash	0.2 M acetic acid in tetrahydrofuran	4
11	Wash	Tetrahydrofuran	4
12	Wash	Dichloromethane	6

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More recently, we assembled a (1→6)-linked triglucosamine (17% overall yield) *(23)*, a branched *Leishmania* tetrasaccharide (α-Man*p*-(1→2)-α-Man*p*-(1→2)-α-[β-Gal*p*-(1→4)]-Man*p*-*O*-4-pentenol, 18% overall yield) *(24)*, and the proteoglycan linkage region tetrasaccharide (β-GlcA*p*-(1→3)-β-Gal*p*-(1→3)-β-Gal*p*-(1→4)-β-Xyl*p*-*O*-4-pentenol) *(25)* employing glycosyl phosphate building blocks in combination with the octenediol linker. In these syntheses, we generally relied on the use of the levulinoyl group for temporary protection and benzyl ethers and pivaloyl esters for permanent protection of the appropriate hydroxyl functions. However, in the assembly of the proteoglycan tetrasaccharide, it was observed that the presence of the bulky C2-pivaloyl ester in the first galactose building block prohibited galactosylation at the C3-OH of this residue. Changing the C2-OH pivaloyl for the smaller acetate group circumvented these problems.

Finally, the Lewis X pentasaccharide **68**, Lewis Y hexasaccharide **69** and Le^y-Le^x nonasaccharide **70** were assembled in an automated fashion, in line with our previous solution phase explorations (*vide supra*). *(26)* For the construction of the challenging oligosaccharide targets five monomeric glycosyl phosphates were selected (Scheme 8A). As compared to the solution phase study, the *N*-phthaloyl trichloroactimidate building block was changed for a *N*-trichloroacetyl phosphate building block, because the latter proved to be more reliable in solid phase assembly. Furthermore, Fmoc and levulinoyl groups were used for

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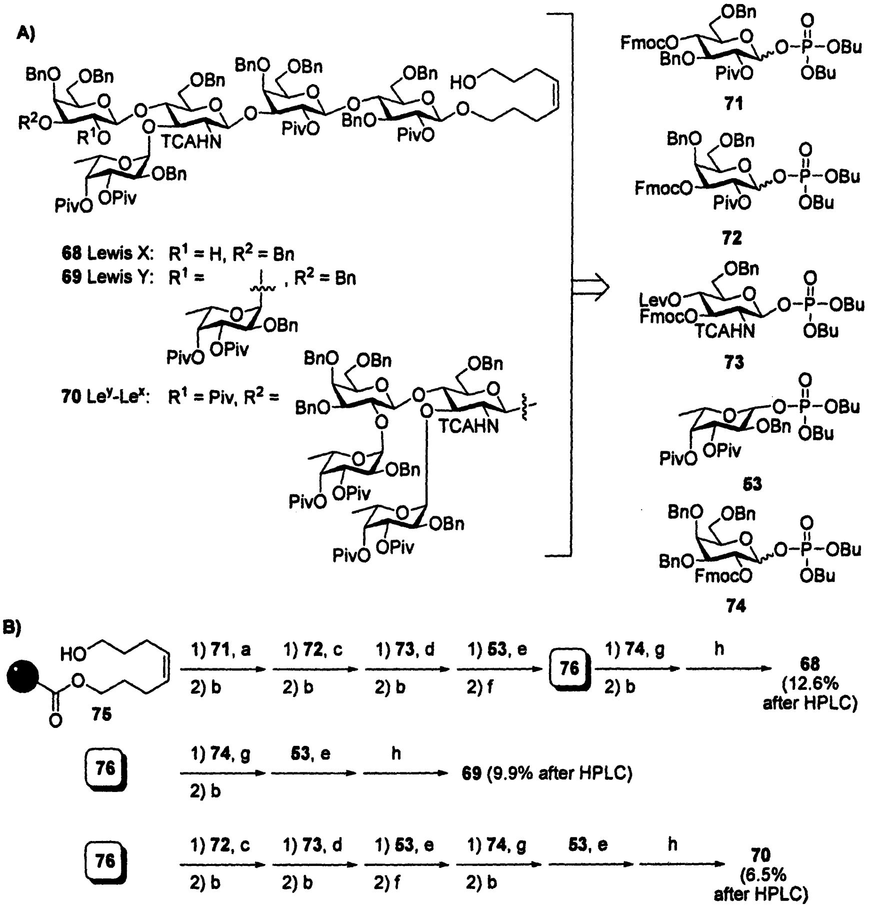
A)
BnO
OBn
R²O
R¹O
TCAHN
PivO
OPiv
HO
68 Lewis X: R¹ = H, R² = Bn
69 Lewis Y: R¹ = , R² = Bn
70 Leʸ-Leˣ: R¹ = Piv, R² =
FmocO
O-P-OBu
OBu
71
72
LevO
73
53
74
B)
75
1) 71, a
2) b
1) 72, c
1) 73, d
1) 53, e
2) f
76
1) 74, g
h
68 (12.6% after HPLC)
53, e
69 (9.9% after HPLC)
70 (6.5% after HPLC)

*Reagents and conditions: Automated synthesis of **68**, **69** and **70** on a 25 µmol scale (45 mg resin, 0.56 mmol/g loading). a) **71** (5 eq.), TMSOTf (5 eq.) repeated 2 times for 15 min. each. b) 2.2 mL of 20% piperidine in DMF solution, repeated 3 times for 10 min. each. c) **72** (5 eq.), TMSOTf (5 eq.) repeated 2 times for 15 min. each. d) **73** (3.5 eq.), TMSOTf (3.5 eq.) repeated 3 times for 15 min. each. e) **53** (5 eq.), TMSOTf (5 eq.) repeated 2 times for 15 min. each. f) 2.5 mL of a 10% N_2H_4 in DMF solution, repeated 5 times for 15 min. each. g) **74** (5 eq.), TMSOTf (5 eq.) repeated 2 times for 15 min. each. h) NaOMe (10 eq.) in $MeOH/CH_2Cl_2$ (1:4), repeated 4 times for 90 min. each.*

Scheme 8. Automated assembly of the blood group oligosaccharides.

temporary protection. A novel octenediol ester linker system (**75**, Scheme 8B), which is fully compatible with the temporary levulinoyl and Fmoc protecting groups, was used. *(27)* Not only is the Fmoc group completely stable under the Lewis acidic conditions used for the coupling and very readily cleaved using mildly basic amines, it also allows for monitoring of the coupling efficiency by UV/VIS spectroscopy, as generally practiced in automated peptide synthesis. New coupling and deprotection cycles were devised for the assembly of oligomers. Initially, the deprotection of the levulinoyl group on the solid support proved troublesome and required modification of the original conditions to improve cleavage efficiency. The activation of the glycosyl phosphates was accomplished at -15°C and two sets of deprotection conditions were employed for Fmoc and levulinoyl cleavage. The complete assembly details are outlined in Scheme 8B.

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Conclusion

Glycosyl phosphates have proven very valuable building blocks for the assembly of complex oligosaccharides. Their ease of synthesis in combination with their rapid activation makes them ideal glycosylating agents to be used for automated solid phase oligosaccharide synthesis.

Acknowledgment

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Chapter 11

Glycosyl Thioimidates as Versatile Glycosyl Donors for Stereoselective *O*-Glycosylation and Convergent Oligosaccharide Synthesis

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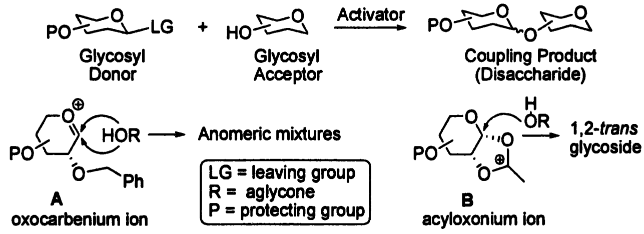
This review discusses the synthesis and application of glycosyl thioimidates as glycosyl donors in stereoselective glycosylation and convergent oligosaccharide assembly. Although a broad introduction and a thorough description of earlier studies of glycosyl thioimidates is presented, the main focus of this review is on S-benzoxazolyl (SBox) and S-thiazolinyl (STaz) derivatives developed by the authors. These novel thioimidates often provide higher stereoselectivity and yields in glycosylations in comparison to other existing approaches. The unique activation conditions of the SBox and STaz glycosides allowed the design of conceptually novel strategies for oligosaccharide synthesis. Such innovative approaches as the inverse armed-disarmed strategy, the O-2/O-5 cooperative effect in glycosylation, the temporary deactivation concept, and orthogonal activation of the STaz vs. S-ethyl moieties are discussed.

In Frontiers in Modern Carbohydrate Chemistry; Demchenko, A.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2007.

Introduction

Elucidation of the exact mechanisms of carbohydrate involvement in pathogenesis of many human diseases is difficult due to the complexity and relatively low availability of natural glycostructures. The majority of the biologically important and therapeutically active carbohydrates are oligosaccharides or complex glycoconjugates (glycolipids, glycoproteins, etc.) in which monosaccharide units are joined via O-glycosidic bonds (*1*). The main conceptual difference between oligosaccharides and other natural biopolymers, proteins and polynucleotides, is in the nature of the intermonomeric bond. The glycosidic bond represents a new chirality center, which often brings along an obstacle associated with the stereoselective synthesis. The necessity to form either a 1,2-cis or a 1,2-trans linkage with complete stereoselectivity is the main reason oligosaccharides remain among the major synthetic challenges of the 21st century. The glycosidic linkage is formed by a glycosylation reaction, most commonly a nucleophilic displacement of a leaving group of the glycosyl donor with a hydroxyl moiety of the glycosyl acceptor (Scheme 1).



Scheme 1. A typical glycosylation reaction

In the case of the ether-type non-participating neighboring substituent, the glycosylation proceeds via the flattened oxocarbenium ion **A** that most commonly leads to the formation of anomeric mixtures (Scheme 1). Although the 1,2-cis glycosides (for D-gluco/galacto series) are favored by the anomeric effect, the stereoselectivity is often low, which makes their synthesis a notable challenge (*2, 3*). The synthesis of 1,2-trans glycosides can be reliably achieved with the use of the neighboring participatory effect of a 2-O-acyl moiety (*4*); these reactions proceed via the bicyclic acyloxonium intermediate **B** with good stereoselectivity.

The development of efficient methods for the synthesis of complex carbohydrates is critical for the field of glycosciences. The glycosylation reaction has already been explored in a number of directions with the main effort focusing on the development of new anomeric leaving groups (*5, 6*). The first controlled and general glycosylation procedure is credited to Koenigs and Knorr

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who accomplished the glycoside synthesis via a nucleophilic substitution at the anomeric center in 1901 (*7*). For many decades this method, in which anomeric bromides and chlorides are used as glycosyl donors, had been the only procedure for the synthesis of a variety of synthetic targets ranging from simple glycosides to relatively complex oligosaccharides (*8*). During the 1970s - early 1980s, a large number of new glycosyl donors were developed: thioglycosides by Ferrier (*9*), Nicolaou (*10*), Garegg (*11*), and others (*12*), cyanoethylidene derivatives by Kochetkov (*13*), O-imidates by Sinay (*14*) and Schmidt (*15*), S-benzothiazolyl derivatives by Mukaiyama (*16*), thiopyridyl derivatives by Hanessian (*17*) and Woodward (*18*), S-pyrimidin-2-yl derivatives by Hanessian (*17*) and Woodward (*18*), and fluorides by Mukaiyama (*19*).

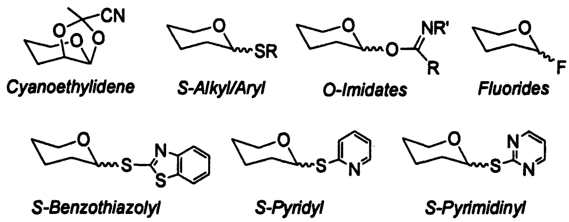

Figure 1. Glycosyl donors introduced in the 1970's - early 1980's

Many glycosyl donors introduced during that period gave rise to excellent complimentary glycosylation methodologies. For example, trichloroacetimidates (*20, 21*), thioglycosides (*22-24*), and fluorides (*25, 26*) are arguably the most common glycosyl donors nowadays. In this respect, glycosyl thioimidates received scarce attention possibly due to the anticipation of only modest glycosyl donor properties and/or marginal stability toward protecting group manipulation. Since the greatest efforts have been directed toward the development of S-alkyl and S-aryl glycoside-based approaches, glycosyl thioimidates have been mainly viewed as a variation of the thioglycoside glycosidation methodology.

Similarly to the alkyl/aryl thioglycosides, glycosyl thioimidates are easily accessible, and could be readily and stereoselectively activated for glycosylation under mild reaction conditions. In contrast to the alkyl/aryl thioglycosides, glycosyl thioimidates can be activated with metal triflates and protic or Lewis acids. In light of these recent discoveries, S-benzoxazolyl (SBox) and S-thiazolinyl (STaz) derivatives demonstrated a number of unique properties. Recent studies determined that certain classes of thioimidates are highly stable toward major protective group manipulations. For example, STaz glycosides were found to be even more stable than conventional thioglycosides. This overview discusses major aspects of the synthesis and application of glycosyl thioimidates in general, and SBox and STaz glycosides in particular, to stereoselective glycosylation and convergent oligosaccharide synthesis.

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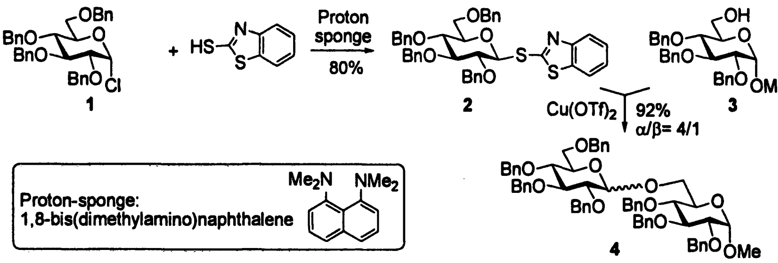
S-Benzothiazolyl derivatives

Per-acetylated S-benzothiazolyl glycosides were first reported by Zinner (*27*). Mukaiyama investigated per-benzylated S-benzothiazolyl glycoside **2** as a glycosyl donor (*16*). Glycosyl donor **2** was prepared from the anomeric chloride **1** and 2-mercaptobenzothiazole in the presence of 1,8-bis(dimethylamino)naphthalene in 80% yield (Scheme 2). Glycosylation studies were performed in the presence of $Cu(OTf)_2$ with various acceptors in diethyl ether and in most cases glycosylations proceeded with high yields and good stereoselectivities. For example, coupling of **2** with glycosyl acceptor **3** in the presence of $Cu(OTf)_2$ provided the disaccharide **4** in 92 % yield as an anomeric mixture ($\alpha/\beta = 4/1$).



Scheme 2. S-Benzothiazolyl approach to glycoside synthesis

A number of improvements to this approach have subsequently emerged. Szeja reported the synthesis of **2** directly from the hemiacetal derivative, 2,3,4,6-tetra-O-benzyl-D-glucopyranose under phase-transfer conditions in the presence of TsCl and Bu_4NCl in benzene/50% aq. NaOH.(*28*). In this case, S-benzothiazolyl glucoside **2** was isolated in 95 % yield as a mixture of α/β-anomers (1/3). Similarly, S-benzothiazolyl mannofuranoside and xylopyranoside were obtained as anomeric mixtures in 92 and 81 % yield, respectively (*28*).

Gama reported glycosidation of **2** by using methyl iodide as an activator in several solvents under high pressure (*29*). It was determined that the high pressure-assisted glycosidation of **2** provided the corresponding disaccharides with an improved stereoselectivity in comparison to that reported by the Mukaiyama group (*16*). For example, coupling of **2** with **3** in dichloromethane at 1.3 GPa afforded **4** in 71 % yield as a mixture of anomers ($\alpha/\beta = 7/1$).

Schmidt and co-workers reported the synthesis of S-benzothiazolyl mannoside **6** by opening the 1,2-orthoester **5** in the presence of $HgBr_2$ (Scheme 3) (*30*). The mannoside **6** was then employed in the intramolecular glycosylation by using *m*-xylylene as a rigid spacer. This was accomplished by protection of

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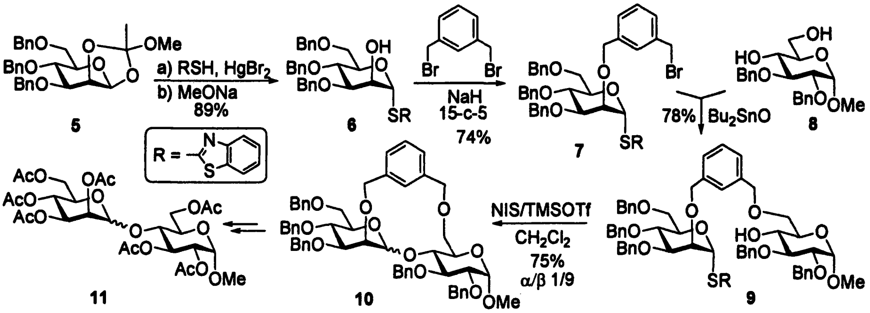
the liberated hydroxyl in **6** with α,α'-dibromoxylene resulting in the intermediate **7**, which was subsequently linked to the glycosyl acceptor **8** to afford **9**. Good β-D-manno stereoselectivity was achieved in NIS-TMSOTf-promoted intramolecular condensation of **9** to afford **10** in 75 % yield (α/β = 1/9), the key step of the synthesis (Scheme 3). The rigid xylylene spacer was then removed by simple hydrogenation to afford **11** upon subsequent acetylation.



Scheme 3. Intramolecular β-mannosylation

Recently, Bogusiak reported the synthesis of S-benzothiazolyl furanosides of the L-arabino, D-ribo, and D-xylo series from the reducing sugars and the 2-mercaptobenzothiazole in the presence of diphenyl phosphoryl chloride [$(PhO)_2P(=O)Cl$] under phase transfer conditions (*31*). It was determined that AgOTf or NIS/TfOH can serve as suitable activators for S-benzothiazolyl furanosides. The coupling products were obtained in moderate to good yields and stereoselectivity. For example, AgOTf-promoted glycosylation of diacetone galactose acceptor (6-OH) with glycosyl donors of the the L-arabino, D-ribo, and D-xylo series in toluene afforded the corresponding disaccharides in 75 % (α/β = 4/3), 98 % (7/2), and 70 % (3/10) yield, respectively.

Ferrieres and Plusquellec described the synthesis of per-acetylated S-benzothiazolyl galactofuranosides from β-D-galactofuranose pentaacetate in the presence of BF_3-Et_2O in 83% yield as an anomeric mixture (*32, 33*). The obtained thioimidates were then deprotected and applied in anomeric phosphorylation. A number of acylated S-benzothiazolyl and S-(5-methoxy)benzothiazolyl derivatives of D-gluco, D-galacto, and D-ribofurano series have been reported by Khodair et al (*34*). While the syntheses of hexoses were accomplished from acetobromosugar and the corresponding sodium thiolates in MeCN, the synthesis of thioribofuranoside derivative was accomplished from ribofuranose tetraacetate and trimethylsilylated thiols in the presence of TMSOTf (*34*).

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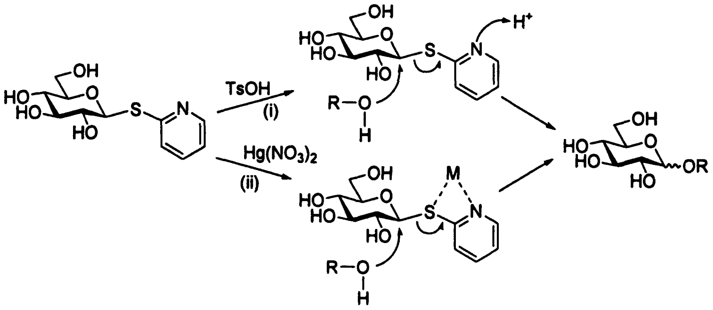
2-Thiopyridyl derivatives

Per-acetylated 2-thiopyridyl glycosides were first prepared from glycosyl halides by Wagner (*35*). Hanessian explored the scope of this class of compounds as glycosyl donors by developing a *"remote activation concept"* (*17, 36*). It was assumed that a suitable substituent at the anomeric center such as sulfur (*soft base*) in combination with a *hard base* (nitrogen) could provide the requirements for the remote activation. This methodology was envisaged for a proton mediated activation process (i), as well as activation by the coordination with a metal ion (ii, Scheme 4). The latter concept was explored by the reaction of unprotected 2-thiopyridyl glycosides with various acceptors in the presence of $Hg(NO_3)_2$ in MeCN (in some cases at reflux). For example, glycosylation of diacetone galactose (6-OH) with unprotected *S*-pyridyl glucoside furnished the corresponding disaccharide in 36% yield as a mixture of α/β-anomers (55/45) (*17*).



Scheme 4. Remote activation of S-pyridyl glycosides

The glycosyl donor properties of protected thiopyridyl glycosides were investigated in the presence of $Hg(NO_3)_2$ (*17*), $Pb(ClO_4)_2$ (*18*), or AgOTf (*37*). However, the preparative application of the approach was significantly enhanced with the introduction of MeI as an activator by Mereyala (*38*). Although in some cases the reaction was rather sluggish and required prolonged reaction times at elevated temperatures, the benefit of high yields and excellent to complete stereoselectivities was apparent. Thus, MeI-promoted coupling between benzylated glycosyl donor **12** and glycosyl acceptor **13** required 3 days at reflux in dichloromethane. Yet, the α(1-4)-linked disaccharide **14** was obtained in a high yield of 82 % and complete α-stereoselectivity (Scheme 5).

The coupling process was anticipated to begin with the *N*-methylation of the S-pyridyl moiety of the glycosyl donor, which was used as an α/β-mixture (the mechanism in Scheme 5 is shown for the β-anomer only). This would lead to the

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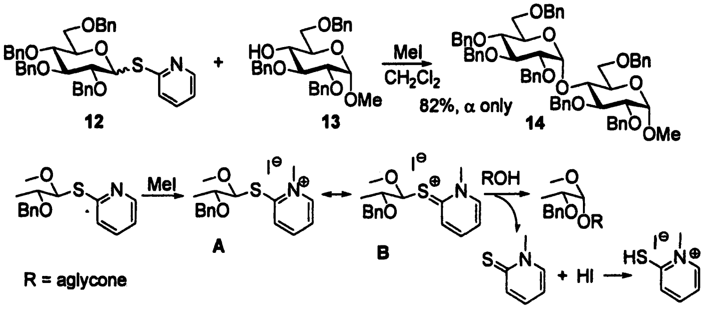
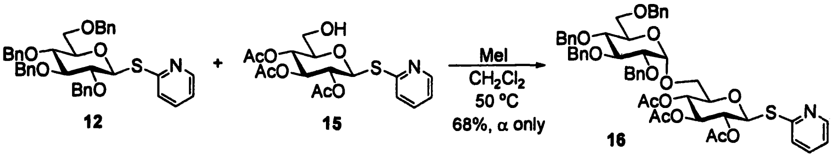
quaternary thiopyridinium salt (β-A), which is further stabilized by the resonance structure β-**B**. Subsequent displacement of the methylated S-pyridyl moiety with a glycosyl acceptor (ROH) possibly occurs in a concerted S_N2 fashion to afford α-glycoside (*39*). Similarly, α-linked donor reacts until the α-sulfonium ion is formed (α-**B**), which first isomerized into β-**B** under the influence of the reverse anomeric effect (*40*), and is then replaced by a nucleophile (glycosyl acceptor).



Scheme 5. Activation of the S-pyridyl glycosides with methyl iodide

The preparative usefulness of the method was also described for the synthesis of 2-deoxyglycosides (*41-44*), furanosides (*45, 46*), aminosugars (*47*), 2-enopyranosides (*48*), and α-fucosides (*49*). One of the most valuable applications of S-pyridyl glycosides is for convergent oligosaccharide synthesis in accordance with the Fraser-Reid's "armed-disarmed" strategy (*50*). Thus, it was demonstrated that the benzylated S-pyridyl glycoside **12** (armed donor) can be chemoselectively activated with MeI over the disarmed (partially acetylated) acceptor **15** in dichloromethane at 50 °C to afford the α-linked disaccharide **16** in 68% yield (Scheme 6) (*42*). The glycosylation step could be reiterated by conversion of **16** into the armed glycosyl donor via sequential deacetylation-benzylation followed by activation with MeI.



Scheme 6. Application of S-pyridyl glycosides in armed-disarmed fashion

To date, the S-pyridyl derivatives are perhaps the most broadly explored glycosyl donors amongst all thioimidates. Other syntheses and applications of

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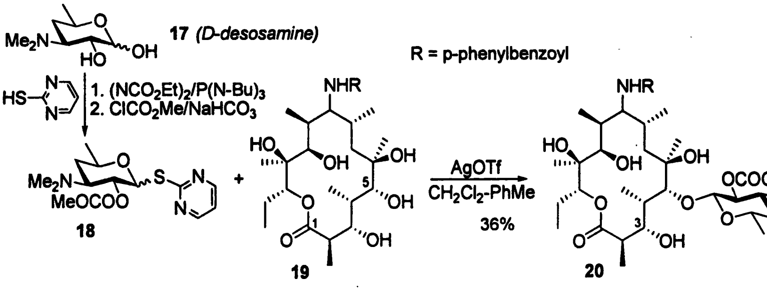
the S-pyridyl glycosides are briefly summarized below. Schmidt and co-workers reported the synthesis and application of S-pyridyl mannoside in intramolecular synthesis of β-mannosides (*30, 51*). Other reports described the synthesis of per-acetylated thiopyridyl furanosides (*32-34*). Szeja and co-workers demonstrated that 5-nitro-2-pyridyl thiomoiety can be used for the anomeric protection of the glycosyl acceptor unit (*52*). Since this anomeric group was found to be stable in the presence of AgOTf, NIS/TfOH, or TMSOTf, a variety of glycosyl donors such as bromides, dithiocarbamates and even S-alkyl glycosides could be selectively activated over the 5-nitropyridyl moiety. Beau developed 2-pyridyl sulfones for the synthesis of C-glycosides (*53*). More recently, Lowary reported the use of this class of glycosyl donors to O-glycosidation of furanoses and pyranoses. Thus, samarium(III) triflate-promoted reactions provided O-linked disaccharides in moderate to excellent yields (*54*). This has become a valuable expansion to the Ley's sulfone approach to O-glycosylation.(*55*).

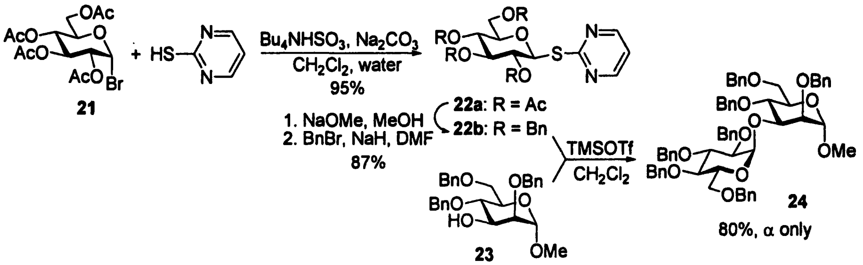
2-Thiopyrimidinyl derivatives

2-Thiopyrimidinyl glycosides were obtained from glycosyl halides by Wagner (*56*). Hanessian investigated these compounds for glycosylation acknowledging qualitatively similar results to those obtained with thiopyridyl derivatives (*17*). Woodward employed 2-thiopyrimidinyl glycosyl donor for the first glycosylation step in the classic synthesis of erythromycin A, a macrolide antibiotic containing two glycosidic linkages (*18*). The glycosyl donor **18** was obtained from D-desosamine **17** and 2-mercaptopyrimidine via n-Bu$_3$P-assisted thioglycosylation followed by the protection at C-2 (Scheme 7). Regioselective glycosylation of the glycosyl acceptor **19** at C-5 with **18** in the presence of AgOTf afforded **20** in 36% yield. Subsequent transformation of **20** involved the glycosylation of the macrolide ring at C-3 with the thiopyridyl donor (*18*). This successful synthesis gave rise to a series of applications of thiopyrimidinyl donors in the syntheses of other glycosylated antibiotics, for instance: oleandomycin (*57, 58*), erythromycin A (*59*), and erythromycin B (*60*).



Scheme 7. Woodward's application of thiopyrimidinyl donor

Arguably the most thorough investigation of the glycosyl donor properties of this class of compounds was performed by Kong and co-workers. Thus, per-benzylated thiopyrimidinyl glycopyranosides of the D-gluco, D-galacto, D-xylo, D-arabino, and L-fuco series have been investigated (*61, 62*). For example, the synthesis of thiopyrimidinyl tetraacetyl-1-thio-β-D-glucopyranoside **22a** was achieved from acetobromoglucose **21** by reaction with 2-mercaptopyrimidine under phase transfer conditions in 95 % yield (Scheme 8).



Scheme 8. S-Pyrimidinyl glycosides in stereoselective 1,2-cis glycosylation

Subsequently, **22a** was converted into the per-benzylated glycosyl donor **22b** by sequential deacetylation and benzylation under standard conditions in 87 % yield. Glycosylation of acceptor **23** with **22b** was performed in the presence of TMSOTf yielding the disaccharide **24** in 80 % yield with complete 1,2-cis stereoselectivity. Ferrieres and Plusquellec described the synthesis of per-acetylated thiopyrimidinyl furanosides and their application to anomeric phosphorylation (*32, 33*).

S-Benzoxazolyl (SBox) derivatives

Per-acetylated SBox derivatives were first synthesized by Zinner from acetobromoglucose and 2-mercaptobenzoxazole (HSBox) in the presence of sodium (*63, 64*). Schmidt and co-workers obtained SBox mannosides by opening 1,2-orthoester derivative **5** with HSBox in the presence of $HgBr_2$ (Scheme 9) (*30*). Demchenko reported the synthesis of the SBox glycosides of the D-gluco, D-galacto, and D-manno series (**27**) from the corresponding anomeric bromides and potassium salt of HSBox (KSBox) in the presence of 18-crown-6 (*65*). Alternatively, glycosyl bromides can react directly with HSBox in the presence of K_2CO_3 in acetone (*66*). In addition, the SBox glycosides were obtained from anomeric acetates (e.g. **25**), chlorides (**28**) (*67*), thioglycosides (**29**) (*68, 69*), or 1,2-anhydro sugars (**26**, Scheme 9) (*67*). Stereoselectivity of the SBox

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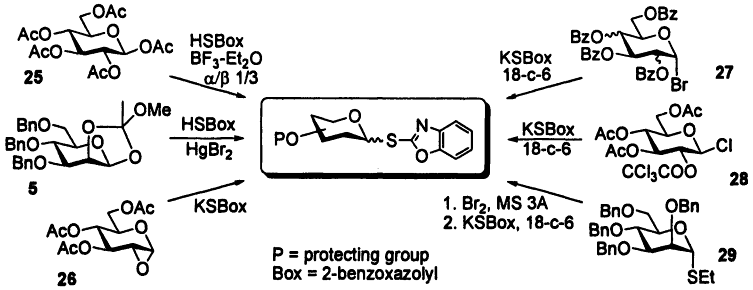
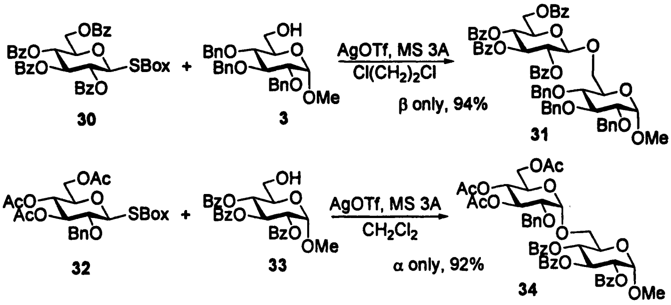
introduction is typically complete, an exception being the BF_3-Et_2O-promoted thioglycosylations of the acetate **25** that result in the formation of considerable amounts of 1,2-cis SBox glycoside (α/β = 1/3) (*65*).


25
HSBox
BF_3-Et_2O
α/β 1/3
5
HSBox
$HgBr_2$
26
KSBox
PO
S
N
O
P = protecting group
Box = 2-benzoxazolyl
KSBox
18-c-6
27
KSBox
18-c-6
28
1. Br_2, MS 3A
2. KSBox, 18-c-6
29

Scheme 9. Synthesis of the SBox glycosides

The application of the SBox glycosides to indirect β-mannosylations, in which glycosyl donor and glycosyl acceptor were linked via a rigid spacer, was reported by Schmidt and co-workers (*30*). The results obtained were in the same range as those described for the S-benzothiazolyl derivatives. Thus, the disaccharide **10** (see Scheme 3) was obtained in 78 % yield (α/β = 1/10). Demchenko et al reported the use of SBox derivatives as glycosyl donors in stereoselective synthesis of 1,2-trans glycosides of the D-gluco, D-galacto, and D-manno series (*65*). Due to the polyfunctional character of the thioimidoyl leaving group, the activation can be achieved via a variety of pathways with the use of AgOTf, $Cu(OTf)_2$, NIS/TfOH, TfOH, or MeOTf. For example, glycosylation of acceptor **3** with glycosyl donor **30** in the presence of AgOTf as a promoter in dichloromethane afforded **31** in 94 % yield (Scheme 10). With the high reactivity of thioimidates, typical glycosidation is complete in minutes.


30
3
AgOTf, MS 3A
$Cl(CH_2)_2Cl$
β only, 94%
31
32
33
AgOTf, MS 3A
CH_2Cl_2
α only, 92%
34

Scheme 10. SBox glycosides in the stereoselective glycoside synthesis

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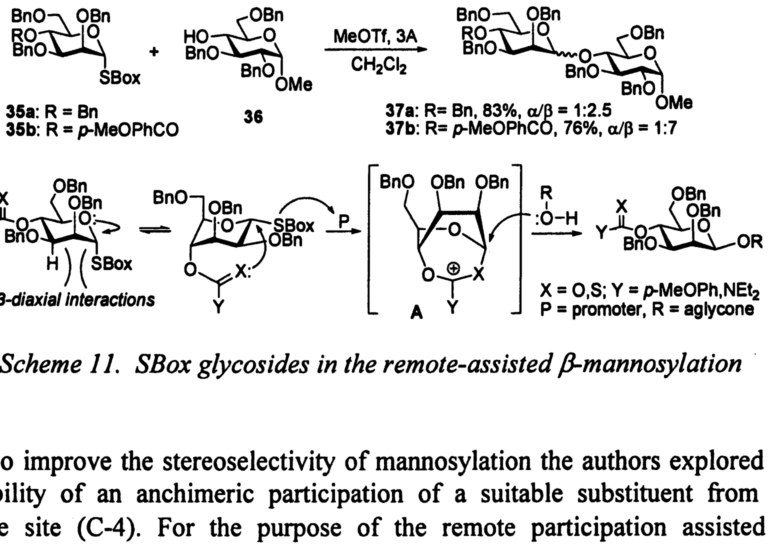
One of the highlights of this methodology has emerged with its evaluation in the synthesis of 1,2-cis glycosides of the D-gluco and D-galacto series (*66*). For example, benzoxazolyl 3,4,6-tri-*O*-acetyl-2-*O*-benzyl-1-thio-β-D-glucopyranoside **32** reacted readily with acceptor **33** in the presence of AgOTf to afford 1,2-cis-linked disaccharide **34** in 92 % yield with complete stereoselectivity (Scheme 10). However, when this methodology was applied to the synthesis of β-mannosides, poor stereoselectivity was recorded in a number of couplings. For example, reaction of **35a** with **36** in the presence of MeOTf led to the formation of a 1:2.5 mixture of α/β-anomers of **37a** in 83 % yield (Scheme 11). This result was not surprising as the synthesis of β-mannosides is a well-known challenge for synthetic carbohydrate chemistry (*70-72*).


BnO
OBn
RO
O
BnO
SBox
+
HO
OBn
O
BnO
BnO
OMe
MeOTf, 3A
CH_2Cl_2
BnO
OBn
RO
O
BnO
OBn
O
BnO
BnO
OMe
35a: R = Bn
35b: R = p-MeOPhCO
36
37a: R= Bn, 83%, α/β = 1:2.5
37b: R= p-MeOPhCO, 76%, α/β = 1:7
X
OBn
OBn
Y
O
BnO
H
SBox
1,3-diaxial interactions
BnO
OBn
O
SBox
OBn
O
X:
Y
P
BnO
OBn
OBn
O
O
X
A
Y
R
:O-H
X
OBn
OBn
O
Y
O
BnO
OR
X = O,S; Y = p-MeOPh,NEt₂
P = promoter, R = aglycone

Scheme 11. SBox glycosides in the remote-assisted β-mannosylation

To improve the stereoselectivity of mannosylation the authors explored the possibility of an anchimeric participation of a suitable substituent from the remote site (C-4). For the purpose of the remote participation assisted β-mannosylation, the SBox glycosyl donors, protected with either *p*-methoxybenzoyl or *N,N*-diethyl thiocarbamoyl moieties at C-4 were obtained (*68*). It was determined that the use of the glycosyl donor **35b** provided significantly higher stereoselectivity in comparison to that obtained with a per-benzylated donor **35a**. Thus, MeOTf-promoted coupling of **35b** with **36** allowed the disaccharide **37b** in 76% yield with notably higher stereoselectivity (α/β = 1/7). Although no thorough mechanistic studies have yet emerged, it has been postulated that the improved stereoselectivity is due to the anticipated long-range participation of a substituent at the remote site C-4. Possibly, this participation would result in the formation of a bicyclic intermediate **A** and, as a result, the nucleophilic attack would be primarily directed from the top face of the ring (Scheme 11).

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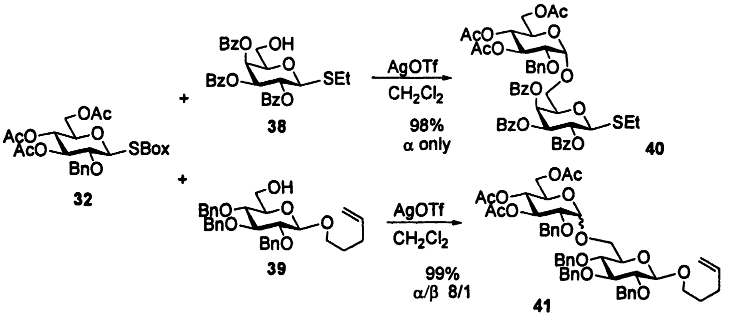
The high reactivity, very high yields, short reaction times, and excellent stereoselectivity are the positive traits of the SBox methodology. Even though these donors belong to the class of thio derivatives, the polyfunctional structure of the SBox moiety enables its activation in the presence of metal triflate-based promoters, such as AgOTf or Cu(OTf)$_2$, which fail to activate the alkyl/aryl thioglycosides.

These factors make SBox derivatives preferable building blocks for selective activation over conventional thioglycosides and other anomeric moieties. For instance, as shown in Scheme 12, the SBox donor **32** could be selectively activated over S-ethyl or *O*-pentenyl glycosides **38** or **39** to give the corresponding disaccharides **40** or **41**, respectively, in high yields (98 - 99%) and stereoselectivities (*65, 66, 73*). Evidently, since the obtained disaccharides already bear a suitable leaving group at the anomeric center, no additional protecting/anomeric group manipulations are necessary for the continuation of the coupling sequence. Further chain elongation can be achieved in a convergent stepwise manner (*65, 66*) or by employing a high throughput one-pot synthesis (*73*).



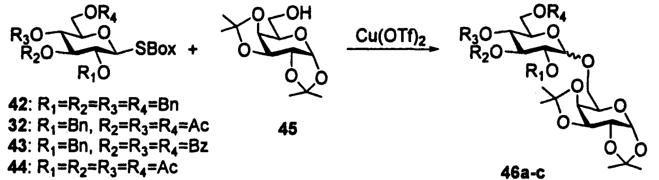
Scheme 12. Selective activation of the SBox glycosides

The applicability of the SBox glycosides to the chemoselective armed-disarmed strategy has been also investigated (*69*). In order to distinguish between armed and disarmed glycosides, the application of a mild promoter, copper(II) triflate, was essential. Thus, activation of **42** over glycosyl acceptor **45** proceeded smoothly, and as a result, the product **46a** was isolated in a good yield of 89% (Entry 1, Table 1) (*69*). When the same reaction conditions were applied to the glycosidation of 2-*O*-benzyl-tri-3,4,6-*O*-acyl protected SBox glucosides **32** and **43**, no product formation was detected.

At the first glance, this result was not a surprise as the lower reactivity could be attributed to the remote disarming effect of the acyl substituents at C-3, C-4, and C-6. Intriguingly, later on it was discovered that supposedly "disarmed" peracylated SBox glycosides **44** and **30**, which were anticipated to be even less reactive than either **32** or **43**, *actually reacted readily in glycosylations.*

Although these glycosylations were marginally slower in comparison to that of the "armed" per-benzylated **42**, they nevertheless proceeded smoothly, yet never went to completion. As a result, disaccharides **46b** and **46c** were isolated in average yields of 69 and 70 %, respectively.

Table 1. Arming and disarming properties of the SBox glycosides



42: $R_1=R_2=R_3=R_4=Bn$
32: $R_1=Bn$, $R_2=R_3=R_4=Ac$
43: $R_1=Bn$, $R_2=R_3=R_4=Bz$
44: $R_1=R_2=R_3=R_4=Ac$
30: $R_1=R_2=R_3=R_4=Bz$

Entry	*Donor*	*Product*	*Yield*	*α/β ratio*
1	**42**	**46a**	89%	5.4/1
2	**32**	--	No reaction	--
3	**43**	--	No reaction	--
4	**44**	**46b**	70%	β-only
5	**30**	**46c**	69%	β-only

The discovered effect was rationalized by a *cooperative O-2/O-5 protecting group effect on glycosylation* (*69*). It was hypothesized that the electronic effects of the C-2 benzyl moiety make the lone pair on the anomeric sulfur *more available* for the promoter attack in **42** in comparison to that in 2-O-benzoylated **30**. However, this does not necessarily mean *faster leaving group departure;* for this, the relative stability of the carbocation intermediates must be also considered. Thus, the glycosyl cation formed from **42** will be stabilized via the O-5 oxocarbenium ion, which will be facilitated if electron-donating groups like benzyls are present in positions O-6 and O-4.

Conversely, if the electron-withdrawing groups are present in C-4 and/or C-6 (as in **32** or **43**), the lone pair on O-5 will be of lower availability, which significantly increases the energy barrier for the formation of the partially acylated oxocarbenium ion. Alternatively, in the case of per-acylated derivatives **44** or **30**, the influence of the remote substituents is diminished by the fact that the formed glycosyl cation can be stabilized by the acyloxonium ion formation (*69*).

The discovered cooperative effect was then applied to convergent synthesis of a variety of differently linked oligosaccharides (*69*). Amongst those, the activation of the *armed* **42** over a *moderately (dis)armed* **47** in the presence of $Cu(OTf)_2$ provided the disaccharide **48** in 64 % yield (Scheme 13). Subsequently, compound **48** was activated over a *disarmed* glycosyl acceptor **49**

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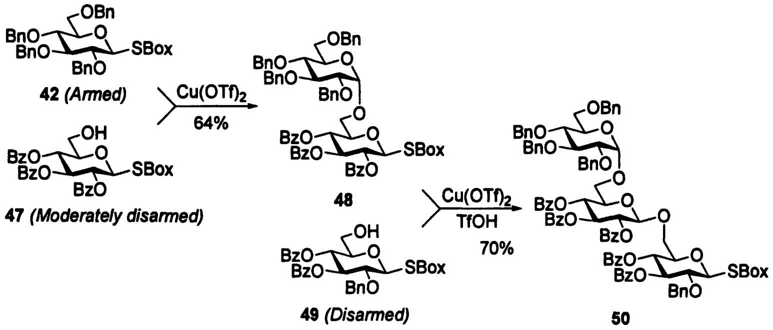
to afford the trisaccharide derivative **50** in 70 % yield. The second activation step required a more powerful activator, $Cu(OTf)_2$-TfOH. Overall, this two-step activation has become direct proof of the observation of the protecting group effect.



Scheme 13. Two-stage chemoselective activation

Recently, De Meo reported the application of the SBox approach to stereoselective α-sialylation and to the convergent synthesis of a GM3 analog (*74*). In the latter synthesis, selective activation of the SBox moiety of the sialosyl donor over the S-ethyl moiety of the galactosyl acceptor was conveniently achieved in the presence of AgOTf. The obtained disaccharide was used in subsequent coupling directly to afford the desired GM3 trisaccharide sequence in good overall yield.

S-Thiazolinyl (STaz) derivatives

Per-acetylated STaz glucopyranosides were first synthesized by Descotes and co-workers from either acetobromoglucose (**20**) and 2-mercaptothiazoline (HSTaz) in the presence of DIPEA or directly from glucose pentaacetate (**24**) and HSTaz in the presence of BF_3-Et_2O in 64 and 69 % yield, respectively (Scheme 14) (*75*). Ferrieres and Plusquellec described the synthesis of per-acetylated thiazolinyl galactofuranosides from β-D-galactofuranose pentaacetate in the presence of BF_3-Et_2O in 53% yield (*32, 33*).

Demchenko reported the synthesis of the benzoylated STaz glycosides from the corresponding anomeric bromides (**27**) and NaSTaz or KSTaz in the presence of a crown ether (*76*). In these syntheses, the STaz glycosides of the D-gluco, D-galacto, and D-manno series were isolated in 60, 90, and 70 % yield, respectively. Subsequently, it was determined that direct conversion of the anomeric acetates into STaz glycosides is by far more efficient for the D-gluco

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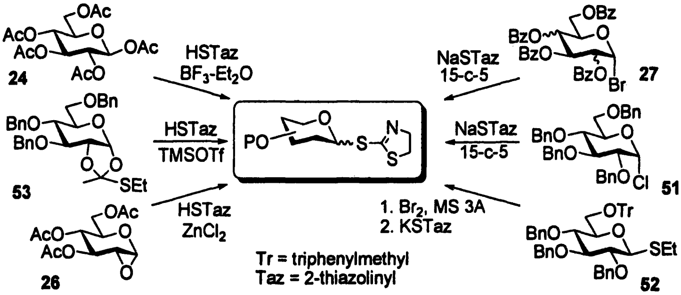
and D-galacto series in comparison to the synthesis from glycosyl bromides. Thus, the target compounds were obtained in 91 and 85 %, respectively (*76*).



Scheme 14. Synthesis of the STaz glycosides

Lower yields in the synthesis of STaz glycosides from glycosyl halides are due to two side reactions, N-glycosylation and β-elimination. Occasionally, the competing processes resulted in notable formation of by-products, N-linked Taz and 1,2-anhydro derivatives, probably due to ambident reactivity of HSTaz and its relatively high basicity. Alternatively, the STaz glycosides were obtained from anomeric chlorides (**51**) (*76*), thioglycosides (e.g. **52**) (*77*), 1,2-anhydro sugars (**26**) (*75*), or 1,2-orthoesters (**53**, Scheme 14) (*78*). The 1,2-trans stereoselectivity of the STaz formation has been complete.

The STaz moiety was found stable toward common protecting group manipulations involving basic and acidic conditions, for example, acetylation, benzylation, acetal formation and cleavage, etc. (*77*). The STaz derivatives were found to be stable toward hydrolysis in the presence of acidic thiophilic reagents. Thus, comparative hydrolytic stability studies showed that STaz glycosides are even more stable than their 1-*S*-ethyl and 1-*S*-phenyl counterparts in the presence of NBS or NIS/TfOH.

Investigation of the glycosyl donor properties of the STaz glycosides resulted in the development of a general approach for 1,2-cis and 1,2-trans glycosylation. Early studies by Descotes and co-workers involved the displacement of the STaz functionality of peracetylated derivatives of the D-gluco series with MeOH in the presence of $HgNO_3$ (*75*). Further expansion of the glycosylation protocol to disaccharide and oligosaccharide synthesis was reported by the Demchenko group. Activators such as AgOTf, MeOTf, NIS/TfOH, or $Cu(OTf)_2$ have been found to be suitable for efficient STaz activation for glycosylation (*76*). It should be specifically highlighted that virtually no glycosylation took place in the presence of NIS in combination with catalytic TfOH, common conditions for thioglycosides activation. In contrast, the reaction was smoothly driven to completion in the presence of NIS and a stoichiometric amount of TfOH.

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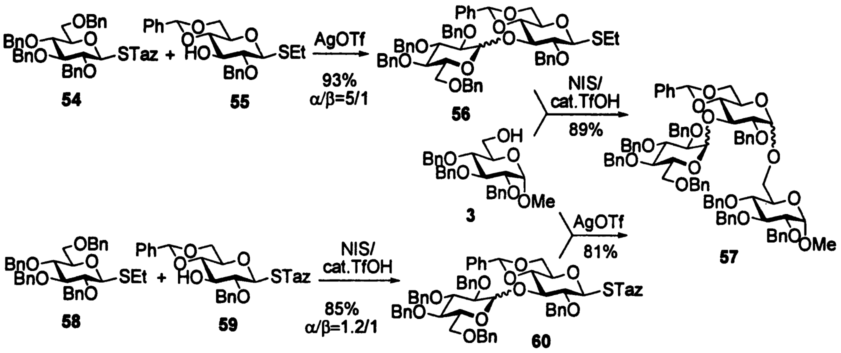
This observation created a solid basis for the evaluation of the STaz glycosides in orthogonal glycosylations in combination with S-ethyl glycosides. The promoter of choice for this synthesis would be AgOTf for the STaz activation, under which conditions the SEt moiety is entirely stable. Conversely, it should be possible to activate SEt in the presence of NIS and catalytic TfOH, conditions under which the STaz moiety remains stable. This concept was demonstrated by synthesizing the trisaccharide **57** via two strategically different approaches.



Scheme 15. Orthogonality of the STaz and SEt glycosides

The first pathway involved activation of the STaz glycosyl donor **54** over SEt glycosyl acceptor **55** with AgOTf to afford the intermediate disaccharide **56**, which was coupled with acceptor **3** in the presence of NIS/cat TfOH to afford **57**. The second pathway involved the glycosidation of the SEt glycosyl donor **58** with STaz acceptor **59** in the presence of NIS/cat. TfOH to afford **60**, which was then activated with AgOTf for the reaction with **3** to afford **57** (Scheme 15).

These syntheses not only demonstrated complete orthogonality of the STaz and SEt glycosides, but also allowed direct comparison of these two types of glycosyl donors. While the yields achieved in these glycosylations were similar for both classes of compounds, the stereoselectivity achieved with STaz glycosides was significantly higher. Thus, **56** was obtained in 93 % yield as a 5/1 mixture of α/β-anomers, while the SEt activation-based synthesis of **60** was achieved in 85% yield with significantly lower stereoselectivity (α/β = 1.2/1).

Additionally, the arming and disarming properties of the STaz glycosides were evaluated. It was demonstrated that activated (benzylated) STaz derivatives could be activated over electronically disarmed (partially acylated) STaz glycosyl acceptors in the presence of either AgOTf or $Cu(OTf)_2$ (*78*). For example, a classic armed-disarmed approach (*79*) allowed chemoselective activation of **54** over disarmed acceptor **61** in the presence of $Cu(OTf)_2$ to afford

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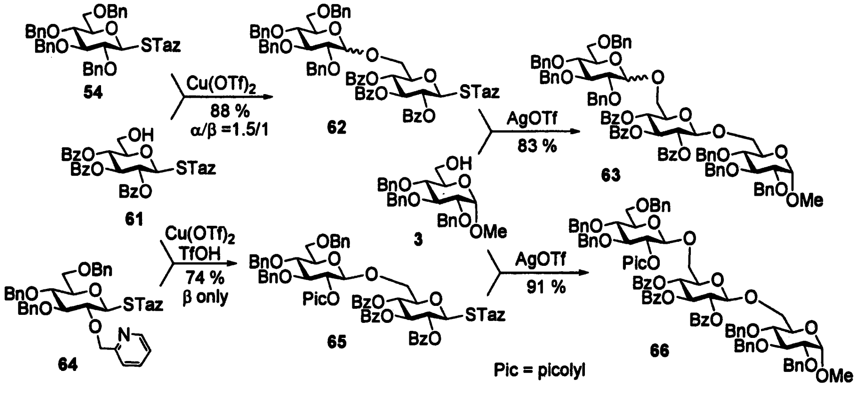
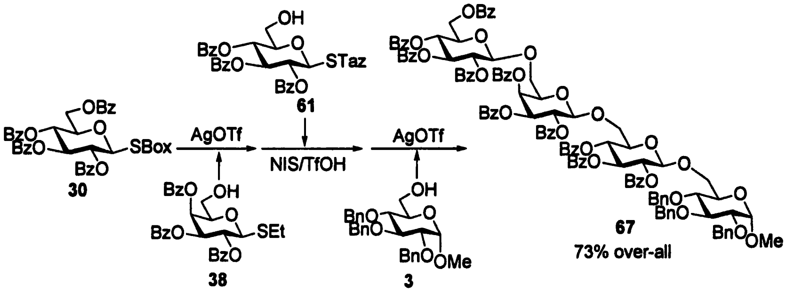
62 in 88 % yield with modest stereoselectivity, yet predominantly as the α-anomer (α/β = 1.5/1, Scheme 16) (*78*). Subsequently, **62** was activated over acceptor **3** with a stronger promoter AgOTf to afford **63**, a trisaccharide with a cis-trans glycosylation pattern.



Scheme 16. Chemoselective activation of the STaz glycosides

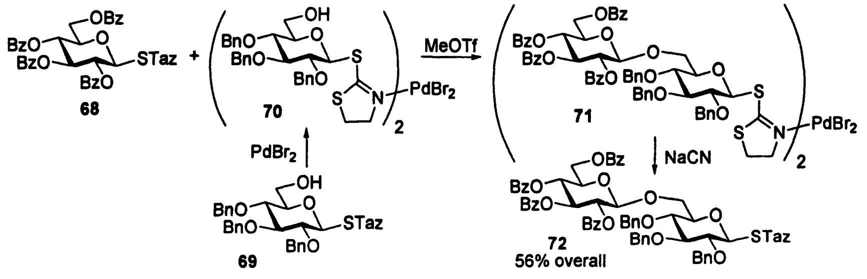
Application of a novel arming participating group 2-O-picolyl in the glycosyl donor **64** provided the disaccharide **65** in 74% yield with complete 1,2-trans stereoselectivity (*78*). Subsequent activation of **65** with AgOTf yielded the trans-trans-linked disaccharide **66** in 91 % yield. Application of this complementary "inverse armed-disarmed" technique is anticipated to be of significant value for convergent oligosaccharide synthesis as it allows introducing a 1,2-trans linkage chemoselectivity prior to other bonds, which would ultimately allow one to access oligosaccharides with the inverse glycosylation pattern (trans-cis) via chemoselective approaches.



Scheme 17. Thioimidate-based one-pot oligosaccharide synthesis

The Demchenko group has also developed a thioimidate-based one-pot glycosylation procedure, which was successfully applied to the synthesis of the tetrasaccharide **67** (*73*). This synthesis involved both SBox and STaz glycosides. Thus, SBox glycoside **30** was activated over SEt acceptor **38** with AgOTf. Upon completion of the coupling, NIS and catalytic TfOH were added to the reaction mixture along with the STaz glycosyl acceptor **61**. In the final step, glycosyl acceptor **3** and AgOTf were added in order to glycosidate the STaz moiety of the trisaccharide intermediate. Upon completion of the one-pot sequence and purification by column chromatography, the tetrasaccharide **68** was isolated in 73 % yield over three steps (Scheme 17).

Demchenko and co-workers have demonstrated that STaz glycosides can participate in stable non-ionizing transition metal complexes. This observation served as a basis for the development of a novel temporary deactivation technique for oligosaccharide synthesis (*77*). The outline of this unprecedented strategy that involves the temporary disarming of a leaving group by external deactivation of its active sites is highlighted in Scheme 18. Thus, the deactivation of the would-be-armed glycosyl acceptor **69** was achieved by engaging its STaz functionality into a stable, non-ionizing metal complex with $PdBr_2$ (**70**). This allowed chemoselective activation of a "free" STaz moiety of the disarmed glycosyl donor **68** over the deactivated (capped) STaz moiety of the complexed acceptor **70** in the presence of MeOTf. Upon glycosylation, the disaccharide **71** was released from the complex by treatment with NaCN to allow "free" disaccharide **72**, which could be used in subsequent transformations.



Scheme 18. Temporary deactivation technique

Recently, De Meo reported application of the STaz approach to stereoselective α-sialylation (*74*). It was determined that STaz sialosides generally provide somewhat lower stereoselectivity in sialylations than their SBox counterparts.

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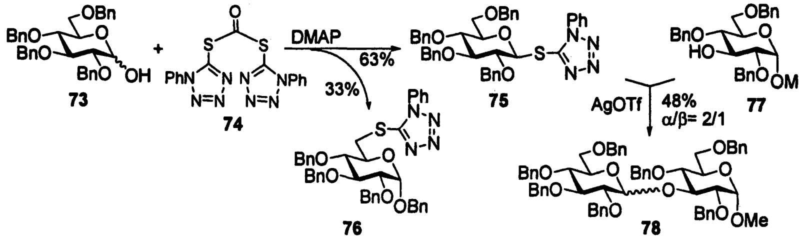
Miscellaneous glycosyl thioimidates

Ogura and co-workers introduced 1-phenyl-1*H*-tetrazol-5-yl thioderivative as a glycosyl donor (*80*). This class of compound was obtained in the perbenzylated form from the corresponding hemiacetal derivative **73** (*80*). Thus, reaction of **73** with S,S'-bis(1-phenyl-1*H*-tetrazol-5-yl) dithiocarbonate **74** in the presence of DMAP in MeCN allowed **75** in 63% yield. The major by-product of this transformation was found to be 6-thio-6-deoxy regioisomer **76** (33%). Glycosylation studies were performed in the presence of AgOTf with various acceptors, which were used in excess (at least 5 mol equiv). For example, AgOTf-promoted glycosylation of **75** with **77** allowed the disaccharide **78** in a fair yield of 48% as a mixture of α,β-anomers (2/1, Scheme 19).



Scheme 19. Investigation of 1-phenyl-1H-tetrazol-5-yl thioderivatives

Hanessian investigated N-methylthioimidazolinyl derivative **79** (Figure 2) for glycosylation acknowledging their "qualitatively similar results" to those obtained with thiopyridyl derivatives (*17*). Schmidt investigated the 2-thiopyrazyl derivative **80** in AgOTf-promoted glycosylations of acceptor **3** (*81*). As a result of these couplings, the disaccharide **4** was isolated in 82 % yield (α/β = 2/1). Schmidt's group also reported the synthesis of 1,3,4-thiadiazole-2,5-diyl bis(thiomannopyranoside) **81** (Figure 2) and its application to the NIS/TMSOTf-promoted indirect β-mannosylation similar to that described for benzothiazolyl thioglycosides (see Scheme 3) (*30*). Ferrieres and Plusquellec described the synthesis of peracetylated S-benzimidazole derivatives of the D-gluco, D-galacto and D-manno furanoside series **82** from the corresponding pentaacetates in the presence of BF_3-Et_2O (*32, 33*).

Demchenko investigated a series of per-acetylated and per-benzoylated thioimidates that include S-oxazolinyl (**83**), S-oxazinyl (**84**), and S-thiazinyl (**85**) derivatives (Figure 2). The glycosyl donor properties of these compounds and their hydrolytic stability were determined to be similar to those of the SBox and STaz glycosides, yet slightly lower glycosylation yields were obtained (*82*). A

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number of other glycosyl thioimidates have been synthesized; however, determination of their glycosyl donor properties has not yet appeared (*83-87*).

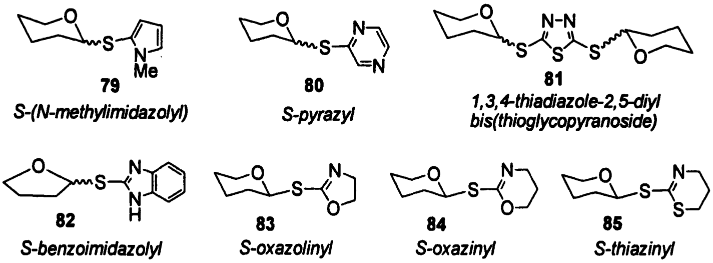

Figure 2. Survey of other glycosyl thioimidates

Conclusions

A number of different classes of glycosyl thioimidates have been discussed herein. Due to a fairly broad diversity of these derivatives, generalization is often impractical. So far, only S-pyridyl, SBox, and STaz glycosides have been more or less systematically investigated. The following conclusions are primarily focused on the properties of the SBox and STaz glycosides developed by the authors of this overview.

Preparation of glycosyl thioimidates. Excellent yields and often complete stereoselectivity were achieved in the syntheses of thioimidates of different carbohydrate series. These compounds can be obtained from a range of precursors, such as acetates, halides, anhydrosugars, hemiacetals, orthoesters with the use of inexpensive aglycones (for example, 2-mercaptothiazoline is even cheaper than thiophenol). The majority of glycosyl thioimidates are prepared from odorless aglycones. Scientists working on glycoside synthesis in biochemistry departments and medical schools with limited fume-hood capability especially appreciate this feature. This, along with other positive traits, will significantly simplify large-scale glycosylations in industry, where the application of thioglycosides is restricted by the necessity to operate with (and dispose of) large quantities of foul-smelling thiols, and the associated by-products.

Glycosidation of glycosyl thioimidates. Excellent yields were often achieved in glycosidation of glycosyl thioimidates. Glycosylation reactions often proceed to completion with no by-product formation and, hence, no significant glycosyl donor excess is typically required. For example, both STaz and SBox glycosides provided very high stereoselectivity in glycosylations, which was achieved with a variety of glycosyl acceptors at room temperature in a neutral solvent

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(dichloromethane or 1,2-dichloroethane). With the high reactivity of thioimidates, typical glycosidation is complete in minutes at room temperature. Although the room temperature activation feature may seem to be insignificant for laboratory use, it becomes essential for the automated and/or energy and labor efficient large-scale industrial applications. Due to the polyfunctional character of the thioimidoyl leaving group, the activation can be achieved via a variety of pathways with the use of AgOTf, $Cu(OTf)_2$, NIS/TfOH, TfOH, or MeOTf. This ultimately allows fine tuning of the activation conditions.

Glycosyl thioimidates in convergent oligosaccharide syntheses. The high stability of thioimidates toward other glycosyl donor activation conditions allows their use for the temporary protection of the anomeric center of the glycosyl acceptor, a facet that is critical for convergent block saccharide synthesis. It has already been determined that both SBox and STaz glycosides follow chemoselective armed-disarmed activation pathway, which is not unusual for stable glycosyl donors. SBox and STaz glycosides can be selectively activated in the presence of other glycosyl donors and, therefore, easily fit into elaborate glycosylation strategies for complex oligosaccharide synthesis. Orthogonality toward other glycosyl donors further increases the value of this approach. Thus, it has been determined that STaz and SEt are entirely orthogonal leaving groups.

A number of other approaches have been developed, amongst which the thioimidate-based one-pot sequential glycosylation strategy has to be specifically highlighted. The fact that glycosyl thioimidates can be engaged in stable non-ionizing transition metal complexes served as a basis for a novel temporary deactivation technique for oligosaccharide synthesis. This unprecedented strategy involves temporary deactivation of the leaving group by external deactivation of its active sites. Other convergent approaches, such as the O-2/O-5 cooperative effect in glycosylation and the inverse armed-disarmed strategy also show a good potential for further development of a well-rounded methodology for oligosaccharide synthesis.

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Chapter 12

Sequential Glycosylation Strategies: A Focus on Thioglycosides as Donors and Acceptors

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This chapter presents an overview on the research on oligosaccharide synthesis at Leiden University, with a focus on chemoselective glycosylations and highlighting the results obtained in the last 5 years.

The development of efficient and widely applicable methods for the synthesis of the structurally diverse class of oligosaccharides is an important challenge in synthetic organic chemistry.[1] In a route of synthesis towards a specific oligosaccharide the following stages can been discerned: 1) the preparation of protected monosaccharide building blocks, 2) the repeated connection of these in a designed strategy using suitable glycosylation procedure(s) and 3) the removal of the protecting groups in the end product to give the target oligosaccharide.

Protecting groups used in the assembly of oligosaccharides not only play a crucial role in attaining the desired regioselectivity, but also exert influence on the reactivity of the applied reaction partners, thereby governing the outcome of the glycosylation in terms of yield and stereoselectivity. Fraser-Reid *et al.*[2] were the first to formulate the influence of protecting groups on the reactivity of donor glycosides. During their investigations on the glycosylation properties of *n*-pentenyl glycosides (Scheme 1), they found that C-2 alkylated *n*-pentenyl

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glycoside **1** (R_1 = *O*-pentenyl) can be condensed chemospecifically under the influence of iodonium di-*sym*-collidine perchlorate (IDCP) with C-2 acylated *n*-pentenyl glycoside **2** (R_1 = *O*-pentenyl), to give *n*-pentenyl disaccharide **3** (R_1 = *O*-pentenyl). This finding is not only the first example of a chemoselective glycosylation, but also led to the formulation of the *armed/disarmed* concept. In this concept, the unreactive C-2 acylated *n*-pentenyl glycosides were termed *disarmed* and their more reactive C-2 alkylated counterparts *armed.*

The armed/disarmed concept proved to be valid for different types of glycosyl donors, as we demonstrated during our studies on the glycosidation properties of thioglycosides.[3] Treatment of a mixture (Scheme 1) of benzylated thioglycoside donor **1** (R_1 = *S*-alkyl or aryl) and partially benzoylated thioglycoside acceptor **2** (R_1 = *S*-alkyl or aryl) with the mild iodonium ion promotor IDCP gave dimer **3** (R_1 = *S*-alkyl or aryl) in 84% yield as a mixture of anomers (α:β = 7:1). Conversion of the acyl protecting groups into alkyl groups makes thioglycoside **3** (R_1 = *S*-alkyl or aryl) amenable for the next chemoselective coupling using IDCP and an acylated thioglycoside acceptor. However, replacement of the benzoyl by benzyl groups in **3** (R_1 = *S*-alkyl or aryl) restricts such a condensation to the formation of predominantly α-linkages. For the objective to introduce β-glycosidic bonds a more potent iodonium ion promotor than IDCP was required. This was found in the *N*-iodosuccinimide (NIS)/trifluoromethanesulfonic acid (TfOH) combination. Treatment of a mixture (Scheme 1) of disarmed dimer **3** (R_1 = *S*-alkyl or aryl) and alcohol R_2OH with the NIS/TfOH combination led to the formation of product **4**, having a β-glycosidic bond at the reducing end.

The glycosylations described above illustrate that the reactivity of thioglycosides is, in part, governed by the electronic properties of the applied protecting groups. The partial positive charge which develops upon departure of the anomeric leaving group is more stabilized in the case of benzyl protecting groups than in the presence of disarming benzoyl groups. Since the effect of protecting groups on the reactivity of donor and acceptor thioglycosides is not only determined by its nature but also by the position on the saccharide core, it is not always easy to predict whether a productive chemoselective glycosylation can be attained. Our studies towards the assembly of a haptenic tetrasaccharide fragment corresponding to the inner cell-wall glycopeptidolipid of *Mycobacterium avivium* illustrates that subtle changes in the protective group pattern of the reacting thioglycosides can have an important influence on the outcome of a chemoselective glycosylation in terms of yield and selectivity.[4]

IDCP mediated coupling (Scheme 2) of 4-O-allyl protected fucopyranoside **5** (R_1 = allyl) with 4-O-benzyl protected rhamnopyranosyl acceptor **6** (R_2 = benzyl) led to the formation of dimer **7** in 57% yield as an anomeric mixture (α:β = 2:1). The rather disappointing outcome of this reaction was ascribed to the formation of 1,2,3-orthoacetate **14**. It was found that replacement of the

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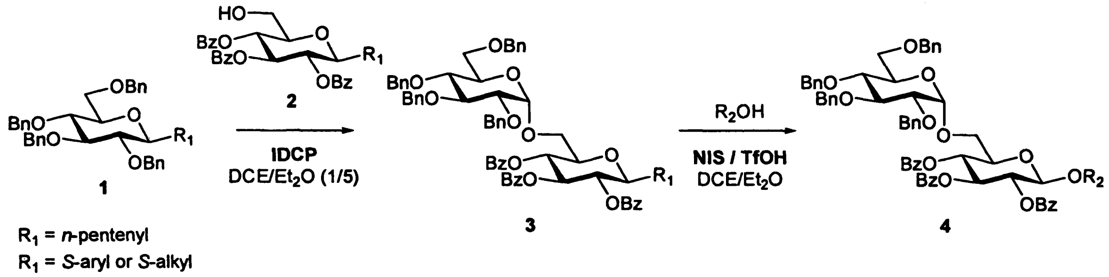
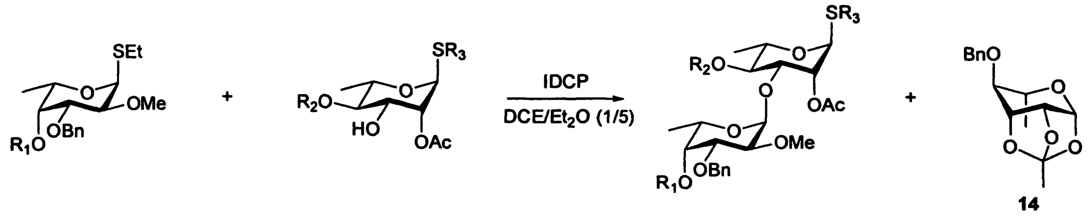

HO
BzO
BzO
R1
OBz
2
BnO
BnO
OBn
O
R1
OBn
1
IDCP
DCE/Et2O (1/5)
OBn
BnO
BnO
BnO
O
BzO
BzO
R1
OBz
3
R2OH
NIS / TfOH
DCE/Et2O
OR2
4
R1 = n-pentenyl
R1 = S-aryl or S-alkyl

Scheme 1


SEt
OMe
OBn
R1O
+
SR3
R2O
HO
OAc
IDCP
DCE/Et2O (1/5)
SR3
R2O
OAc
OMe
OBn
R1O
+
BnO
14

5: R_1 = allyl	**6**: R_2 = benzyl, R_3 = ethyl	**7**: 57% α:β=2:1
5: R_1 = allyl	**8**: R_2 = benzoyl, R_3 = ethyl	**9**: 80% α:β=2,5:1
10: R_1 = chloroacetyl	**8**: R_2 = benzoyl, R_3 = ethyl	**11**: 65% α:β=1:0
10: R_1 = chloroacetyl	**12**: R_2 = benzoyl, R_3 = phenyl	**13**: 80% α:β=1:0

Scheme 2

benzyl group in compound **6** by a benzoyl group (**8**) suppressed cyclization to compound **14** and increased the yield to 80% (dimer **9**). As the moderate stereochemical outcome of both condensations was attributed to the presence of a non-participation group at C-2 and C-4, the chloroacetyl group was introduced at the C-4 position of the donor (**10**, R_1 = chloracetyl). Reaction of donor **10** with acceptor **6** (R_2 = benzyl) mainly afforded cyclization product **14**, while condensation of compound **10** with the less reactive **8** (R_2 = benzoyl) gave access to the pure α dimer **11** in 65% yield. The yield could even be increased (80%) by replacement of the anomeric thioethyl function in compound **8** by the thiophenyl group (**12**, R_3 = phenyl).

Chemoselective glycosylations using thioglycosides and IDCP or NIS/TfOH as activator are restricted to donor and acceptor combinations in which the donor is sufficiently more reactive than the acceptor. Therefore, a productive chemoselective coupling in which both donor and acceptor are armed (or disarmed) cannot be attained. On the basis of the finding that thioethyl glycosides are slightly more reactive than the corresponding thiophenyl glycosides attention was focused on modulating the reactivity of thioglycosides by the use of different anomeric functions that are sensitive to IDCP or NIS mediated activation. The report of Pinto *et al.*[5] on the increased reactivity of selenoglycosides as compared to the corresponding thioglycosides guided us to execute the set of reactions presented in Scheme 3.[6]

Benzylated selenogalactopyranoside **15** could be coupled under influence of IDCP with the less armed thioglucopyranoside **16** to give the expected dimer **17** in a yield of 79%. The NIS/TfOH mediated condensation of benzoylated selenogalactopyranoside donor **18** and the more disarmed thioglucopyranoside acceptor **19** led to the isolation of dimer **20** in the same yield. However, condensation of benzoylated selenophenyl donor **18** with benzylated thioethyl acceptor **16** was unsuccessful and the anhydro derivative **21**, produced by the cyclization of acceptor **16** was isolated as the exclusive product.

Alternatively the nucleophilicity of the anomeric sulfur atom in phenylthio glycosides can be varied by introduction of electron withdrawing or donating substituents in the phenyl ring. The group of Roy developed the use of 4-nitrophenyl thioglycosides as valuable building blocks in their active-latent strategy to prepare oligosaccharides.[7] Scheme 4 presents our efforts on the iodonium ion promoted glycosylations using 4-nitrophenyl thioglycosides as acceptors.[8] Less armed acceptor **23** could be coupled under influence of IDCP with benzylated thioethyl donor **22** to give dimer **24** in a low yield and disappointing anomeric ratio. A more favorable outcome was obtained in the NIS mediated condensation of benzoylated thioethyl donor **25** with the more disarmed acceptor **26** to give dimer **27**. Using identical conditions dimer **28** was

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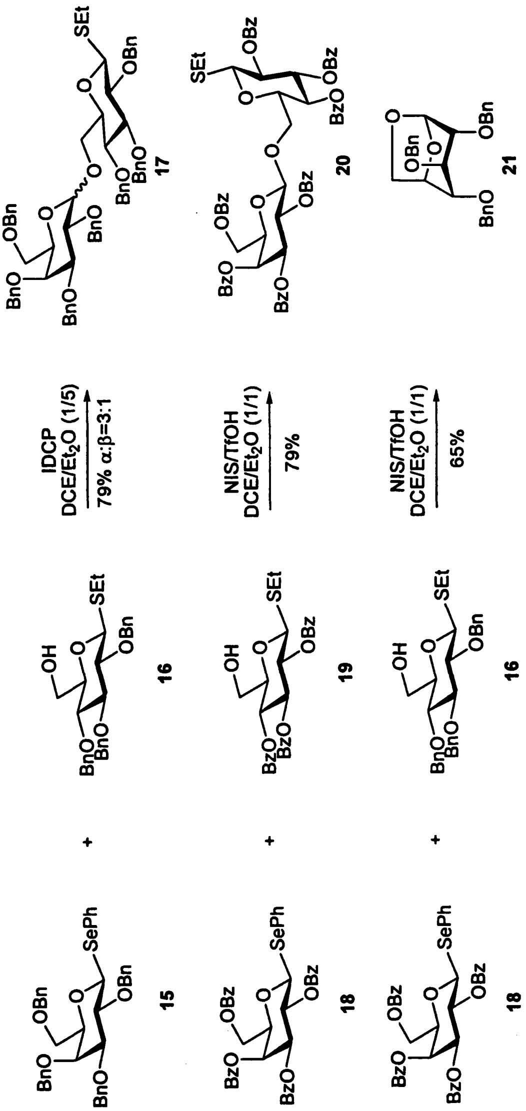
15
16
17
18
19
20
21
SePh
SEt
BnO
OBn
BzO
OBz
OH
IDCP
DCE/Et$_2$O (1/5)
79% α:β=3:1
NIS/TfOH
DCE/Et$_2$O (1/1)
79%
65%

Scheme 3

obtained in a low yield by reaction of the disarmed thioglucopyranoside donor **25** with the benzylated nitrophenyl thioglucoside **23**. Surprisingly the regular IDCP mediated condensation of benzylated thioethyl donor **22** with benzoylated nitrophenyl thioglucoside **26** led to the formation of the corresponding dimer in a low yield. (data not shown). The low yield of the glycosylation reactions described can partly be ascribed to the activation of the nitrophenyl acceptors **23** and **26**, resulting in the cyclization to corresponding 1,6-anhydro derivatives. On the other hand the observed inertness of the individual acceptors **23** and **26** toward both IDCP and NIS/TfOH indicates that the formation of 1,6-anhydro sugars, accompanying the glycosylations recorded in Scheme 4, cannot be solely ascribed to a direct activation of the 4-nitrophenylthio acceptors by the iodonium promoters. A reactive species, generated *in situ* upon activation of the respective ethylthio glycosyl donors by iodonium ions may account for this side reaction.

The observation that iodonium ion promoted glycosylations can be accompanied by the formation of unidentified reactive species which negatively influence the outcome of the condensation reactions in terms of yield as well as chemo- and stereoselectivity guided us to explore other thiophilic activator systems. Our attention was attracted to the 1-benzenesulfinyl piperidine (BSP)/trifluoromethanesulfonic anhydride (Tf_2O) combination from the Crich laboratory that proved to be efficient in the activation of both armed and disarmed thioglycosides in non-chemoselective glycosylation events.[9] In particular, the BSP/Tf_2O system was applied to introduce the highly demanding β-mannosidic linkage. Treatment of the 4,6-O-benzylidene protected mannopyranoside **29** with BSP/Tf_2O led to the formation of the α-anomeric triflate **30**, which is stabilized by the torsionally disarming benzylidene function (Scheme 5). Upon addition of the acceptor **31** the axial triflate in **30** is thought to undergo an S_N2-type displacement, producing the β-mannoside **32** with a high degree of stereospecificity.

As part of a program to assemble the repeating unit of the acidic polysaccharide of the bacteriolytic complex of lysoamidase we first explored the effectiveness of the BSP/Tf_2O promotor combination for the stereoselective introduction of 2-azido-2-deoxy-β-D-mannopyranoside linkages.[10] Subjection of phenylthio donor **33** (Scheme 6) to the standard BSP/Tf_2O protocol at –78°C did not lead to reproducible results. It appeared that complete activation of thiophenyl donor **33** could not always be attained at that temperature and in some pilot experiments the 2-azidoglucal, originating from abstraction of the C-2 proton in the transient contact ion pair, could be isolated. As the reactivity of donor **33**, in comparison with the corresponding mannopyranosyl donor **29**, was decreased by the electron withdrawing effect of the 2-azido group, it was reasoned that activation of a 2-azido mannopyranoside donor can be effected either by the use of a more powerful thiophilic promotor or by enhancement of the nucleophilicity of the anomeric sulfur atom. The latter could be reached by

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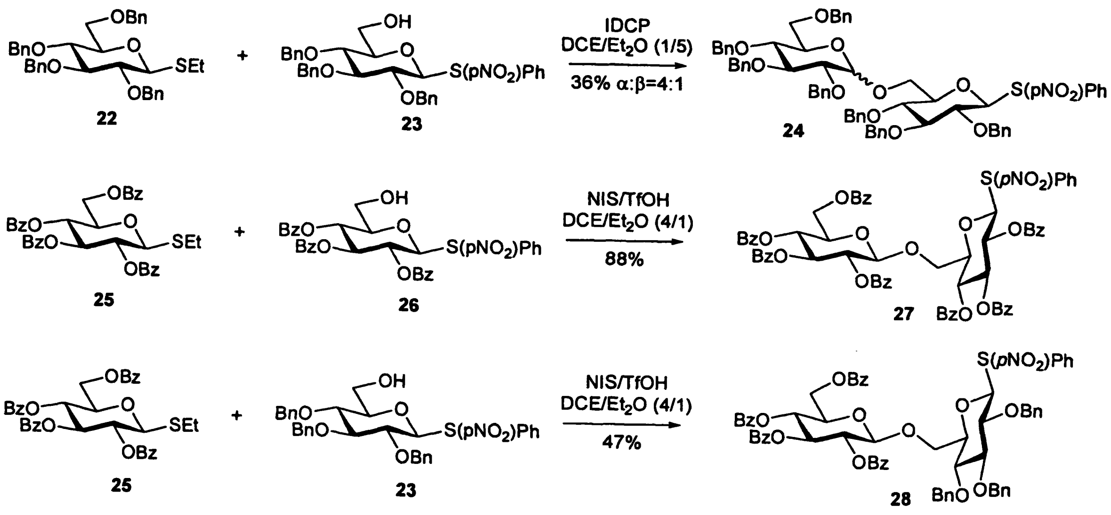
22
23
IDCP
DCE/Et$_2$O (1/5)
36% α:β=4:1
24
25
26
NIS/TfOH
DCE/Et$_2$O (4/1)
88%
27
25
23
NIS/TfOH
DCE/Et$_2$O (4/1)
47%
28

Scheme 4

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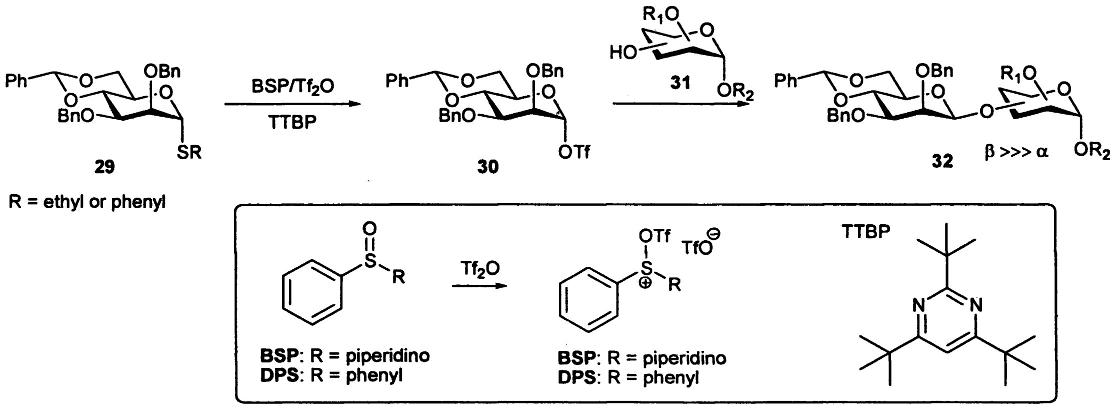
Ph
O
O
BnO
OBn
O
SR
29
R = ethyl or phenyl
BSP/Tf2O
TTBP
OTf
30
R1O
O
HO
31
OR2
32
β >>> α
OR2
BSP: R = piperidino
DPS: R = phenyl
Tf2O
OTf
TfO⊖
S
⊕
R
BSP: R = piperidino
DPS: R = phenyl
TTBP
N
N

Scheme 5

replacement of the anomeric thiophenyl group by the more electron donating *p*-methoxyphenylthio group (Scheme 6).

Indeed, activation of *p*-methoxyphenylthio donor **34** using the standard BSP/Tf_2O protocol and subsequent addition of a suitable acceptor led to the stereoselective formation of β-linked 2-azido-2-deoxy-D-mannoside **35**. Alternatively, application of the more powerful activator system diphenylsulfoxide (DPS)/Tf_2O in combination with the slightly less reactive thiophenyl donor **33** gave comparable results as illustrated by the stereoselectivities and yields obtained by the glycosylations toward disaccharides **36**, **37** and **38** (Scheme 6). The increased thiophilicity of the DPS/Tf_2O combination can be explained by the fact that the stabilizing effect of the piperidine nitrogen lone pair on the sulfur cation as present in the BSP/Tf_2O combination, is lacking in the DPS/Tf_2O combination.

The difference in thiophilicity between the BSP/Tf_2O and DPS/Tf_2O activator systems, the broad variety of thioglycosides in terms of reactivity that can activated with these systems together with the finding that after activation an intermediate anomeric triflate is formed stimulated us to explore whether these activators could be used in a chemoselective strategy.[11] In the first instance the stepwise protocol, as developed by Crich and coworkers was set aside, and it was investigated whether treatment of a 1:1 mixture of a thio donor and an acceptor having an inert anomeric *O*-methyl function with one equivalent of the BSP/Tf_2O activator in the presence of the base 2,4,6,tri-tert-butylpyrimidine (TTBP) would lead to a productive coupling. It appeared that the thio donor remained unaffected, while the acceptor was converted into an *O*-benzene-(*N*-piperidinyl)sulfonium triflate adduct. Obviously, the formation of the intermediate glycosyl triflate can only be attained by the stepwise protocol of Crich, entailing premixing of the donor thioglycoside and the sulfonium triflate and subsequent addition of the acceptor and TTBP. However, adopting this protocol for the chemoselective condensation of armed thioglucoside donor **22** and disarmed thioglucoside acceptor **19** an intractable mixture of compounds was obtained. Monitoring the progress of this reaction at -60^0C showed that after activation of **22** the intermediate triflate **39** reacted smoothly with thioethyl acceptor **19** to give dimer **41** (Scheme 7).

It was reasoned that upon warming of the reaction mixture to room temperature, disaccharide **41** degraded in the presence of the transiently formed (*N*-piperidinyl)phenyl(*S*-thioethyl)sulfonium triflate **40**, generated upon activation of the anomeric thioethyl function in **22** with BSP/Tf_2O. The assumption that sulfonium triflate **40** is capable of activating thioglycosides at higher temperatures was confirmed by pilot experiments in which a thiophenyl analogue of the thioethyl triflate **40** was independently prepared and examined on its capacity to activate thioglycosides. This reagent proved to be substantially less effective than the BSP/Tf_2O combination and for this reason attention was

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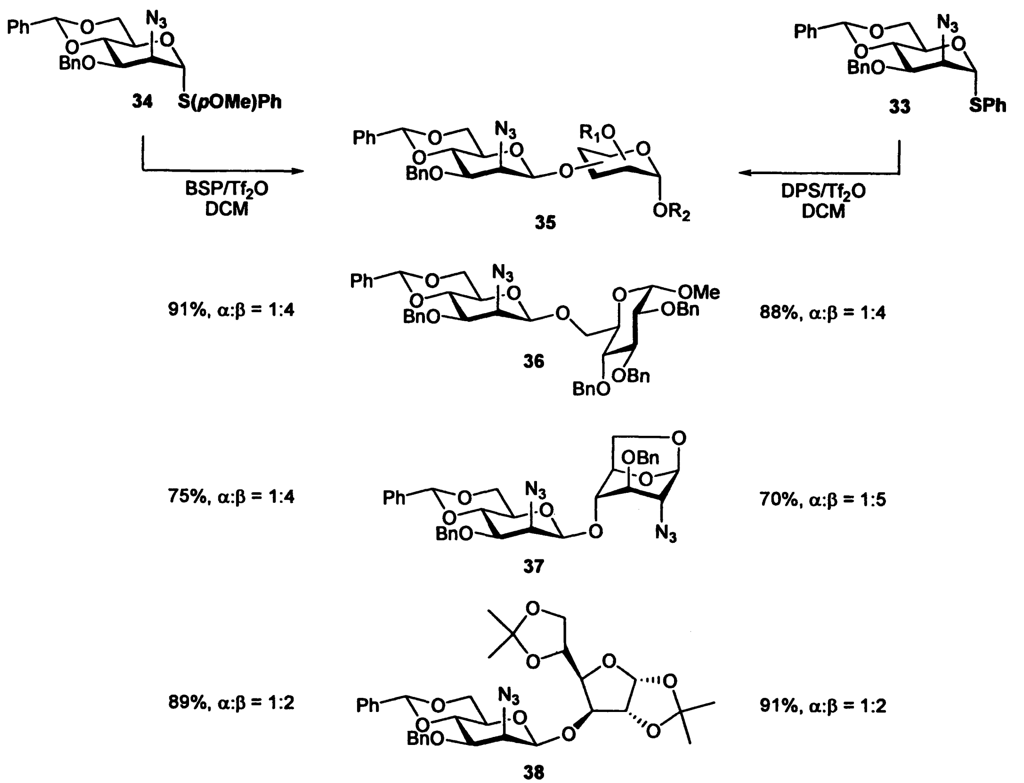
Ph
O
O
BnO
N_3
O
34
S(pOMe)Ph
BSP/Tf_2O
DCM
R_1O
O
OR_2
35
33
SPh
DPS/Tf_2O
DCM
91%, α:β = 1:4
88%, α:β = 1:4
OMe
OBn
OBn
BnO
36
75%, α:β = 1:4
70%, α:β = 1:5
OBn
37
89%, α:β = 1:2
91%, α:β = 1:2
38

Scheme 6

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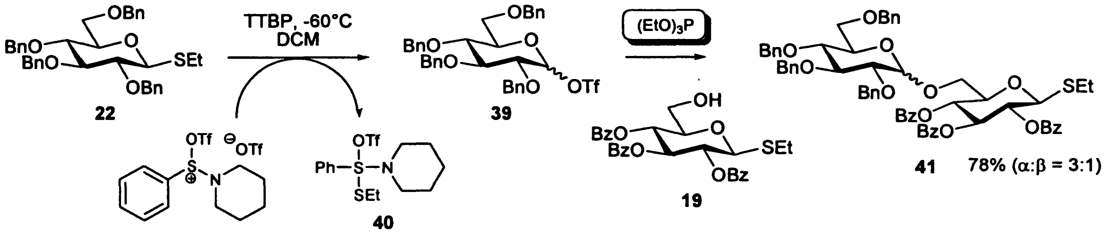
OBn
BnO
BnO
O
SEt
OBn
22
TTBP, -60°C
DCM
OTf
⊖OTf
S
⊕
N
OTf
Ph—S—N
SEt
40
OBn
BnO
BnO
O
BnO
OTf
39
(EtO)₃P
OH
BzO
BzO
O
SEt
OBz
19
OBn
BnO
BnO
O
BnO
O
BzO
BzO
O
SEt
OBz
41
78% (α:β = 3:1)

Scheme 7

focused on scavenging the transiently formed **40**, after completion of the condensation reaction. Earlier studies from our laboratory revealing the favorable properties of triethylphosphite $(EtO)_3P$ as scavenger in the trimethylsilyl triflate mediated chemoselective condensations of arylsulfenyl glycosides guided us to explore this reagent.[12] After it had been established that the decomposition of the anomeric thio function by the sulfonium triflate **40** could be prevented by the addition of triethylphosphite, the BSP/Tf_2O mediated glycosylation of **22** with **19** was revisited. Pre-activation of armed thio donor **22** with BSP/Tf_2O at -60^0C, addition of the disarmed acceptor **19**, was followed by warming up the reaction mixture to -10^0C and addition of one equivalent triethylphosphite. The thiodisaccharide **41** was isolated in 78% yield as a mixture of anomers.

The successful condensation of fully benzylated donor **22** with partially benzoylated acceptor **19** was the starting point to examine a range of differently protected thioglycosides with this glycosylation protocol (Scheme 8). For instance, armed thiogalactopyranoside **42** was chemoselectively coupled to the disarmed azido acceptor **43**, producing the α-linked disaccharide **44** in 73% yield. The condensation of thioglucoside **45** with azido glucoside **46** led to a higher yield (**47**, 90%) but lower stereoselectivity. Fully benzoylated galactopyranoside **48** was selectively condensed with the more disarmed acceptor **46** yielding **49** in 64% yield.

The assumption that the BSP/Tf_2O mediated activation of a thioglycoside donor leads to the formation of the corresponding anomeric triflate together with the possibility of removing thiophilic by-products such as triflate **40** with triethylphosphite could mean that this chemoselective glycosylation protocol is independent of the protective group pattern in donor and acceptor molecules. However, condensation of armed donor **42** (R_1 = benzyl) with armed acceptor **50** (R_2 = benzyl) gave complex reaction mixtures with the 1,6-anhydro derivative of the acceptor as the main compound. This probably originates from transfer of the activated species from the donor to the acceptor followed by intra-molecular attack. Changing the benzyl-groups for benzoyl-groups in both donor and acceptor also proved to be non-productive in a series of pilot experiments in which activation time and quenching temperatures were varied. Strikingly, in some cases the donor molecule (**48**) was recovered, which should be an indication for incomplete conversion of the activated donor to the α-triflate.

From the above can be concluded that BSP/Tf_2O mediated chemoselective glycosylations are restricted to the use of a set of donors and acceptors, in which the donor is more reactive toward the sulfonium activator than the acceptor. Next, attention was focused on the elongation of the obtained thio dimers with the more potent DPS/Tf_2O combination. An example, the assembly of the fully protected α–Gal epitope **55**, is portrayed in Scheme 9. The second DPS/Tf_2O

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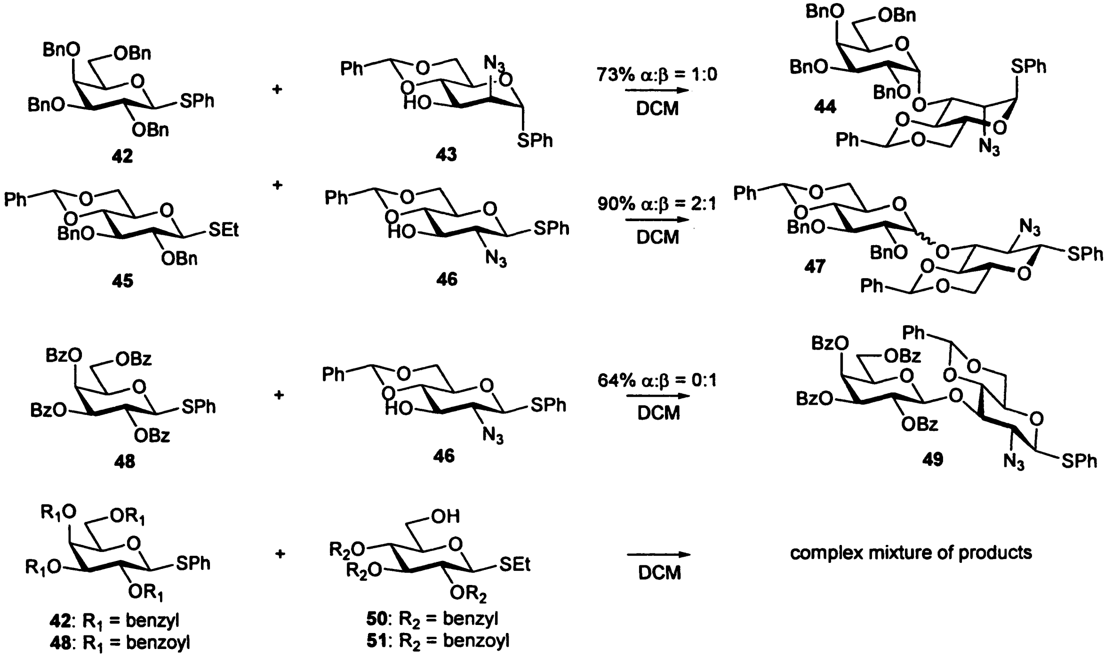
42
43
73% α:β = 1:0
DCM
44
45
46
90% α:β = 2:1
DCM
47
48
46
64% α:β = 0:1
DCM
49
DCM
complex mixture of products
42: R₁ = benzyl
48: R₁ = benzoyl
50: R₂ = benzyl
51: R₂ = benzoyl

Scheme 8

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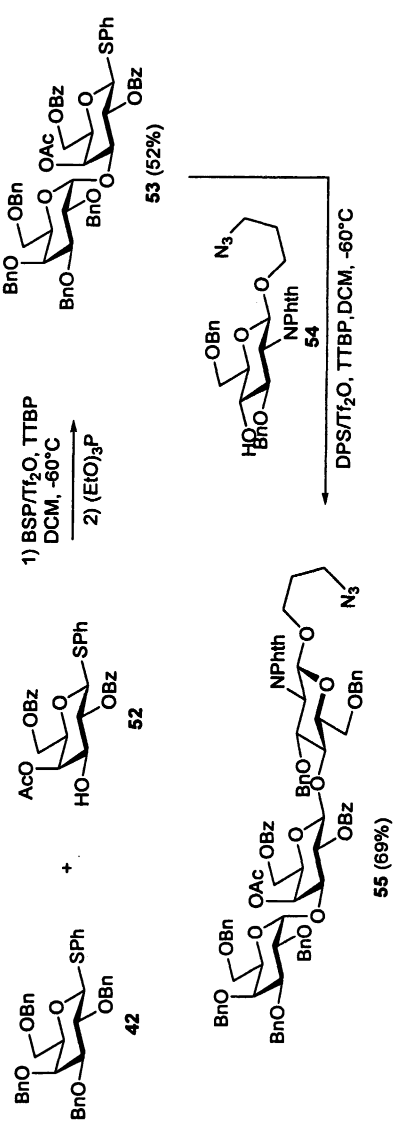
BnO
OBn
BnO
O
SPh
OBn
42
+
AcO
OBz
HO
O
SPh
OBz
52
1) BSP/Tf2O, TTBP
DCM, -60°C
2) (EtO)3P
BnO
OBn
BnO
O
BnO
O
OAc
OBz
O
SPh
OBz
53 (52%)
OBn
HO
BnO
O
NPhth
O
N3
54
DPS/Tf2O, TTBP,DCM, -60°C
BnO
OBn
BnO
O
BnO
O
OAc
OBz
O
OBz
BnO
O
NPhth
O
OBn
O
N3
55 (69%)

Scheme 9

mediated coupling of the disarmed disaccharide **42** with the terminal glucosamine **52** proceeded stereoselectively in 69% yield.

The DPS/Tf_2O combination stems from the dehydrative glycosylation procedure as developed by the laboratory of Gin.[13] In their studies it was shown that pre-activation of a 1-hydroxy donor (such as **56**) with DPS/Tf_2O and subsequent condensation with a suitable alcohol leads to the expected glycoside and concomitant regeneration of DPS. Since both 1-hydroxyl and 1-thioglycosides can be activated by the same sulfonium reagents, it was investigated whether these procedures can be combined to a novel glycosylation strategy, in which a 1-hydroxy donor is condensed with a thioglycoside acceptor yielding a thiodisaccharide, amenable for further elongation using the same sulfonium activator.[14] In the original protocol of the group of Gin a hydroxyl donor (such as **56**) was condensed under influence of an excess of DPS/Tf_2O activator with an excess of a suitable acceptor. We reasoned that adaptation of this protocol is needed to circumvent the unwanted activation of the thioglycoside acceptor by the excess of DPS/Tf_2O. The activation of the thioglycoside acceptor could be completely suppressed by the use of 1.1 equivalent of DPS/Tf_2O activator with respect to the 1-hydroxyl donor, which in turn is employed in excess to the thioglycoside acceptor. Application of this protocol to the assembly of disaccharide **58** proved to be successful (Scheme 10). Treatment of benzylated 1-hydroxyl donor **56** with DPS/Tf_2O in the presence of TTBP and condensation with the secondary hydroxyl of benzylated thioglucoside acceptor **57** gave thiodisaccharide **58** in 87% yield (α:β = 3:1).

The protocol leading to compound **58**, in which both donor and acceptor were armed, was followed by a more demanding condensation in which the 1-hydroxyl donor is disarmed, while the thiophenyl acceptor is armed (Scheme 10). DPS/Tf_2O mediated reaction of pivaloylated donor **59** with thiomannopyranoside **60** mainly gave the corresponding orthoester. Decreasing the amount of TTBP base to 1.5 equivalents sufficiently suppressed orthoester formation and the expected disaccharide **61** was isolated in 80% yield.

The outcome of the glycosylation reactions, recorded in Scheme 10 indicates that the sulfonium ion mediated condensation of 1-hydroxyl donors with 1-thio acceptors is independent of the protecting group pattern of the reaction partners. The fully protected α–Gal epitope **55**, containing an azidopropyl spacer at the reducing end was selected as target to investigate whether this procedure could be extended to the synthesis of trisaccharides (Scheme 11). DPS/Tf_2O mediated dehydrative glycosylation of benzylated galactose donor **62** and acylated thiogalactose acceptor **52** gave the α–linked disaccharide **53** in 64% yield. The DPS/Tf_2O activator combination was also used to couple the disarmed thio disaccharide **53** with glucosamine **54** to afford target trisaccharide **55** in 69% yield. This sequential glycosylation strategy was implemented in the synthesis of a pentasaccharide fragment of heparin.[15]

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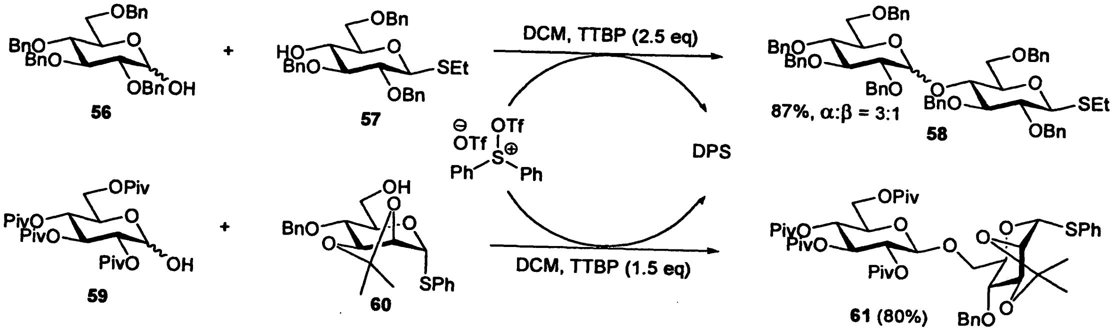
56
57
DCM, TTBP (2.5 eq)
87%, α:β = 3:1
58
DPS
59
60
DCM, TTBP (1.5 eq)
61 (80%)

Scheme 10

In Frontiers in Modern Carbohydrate Chemistry; Demchenko, A.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2007.

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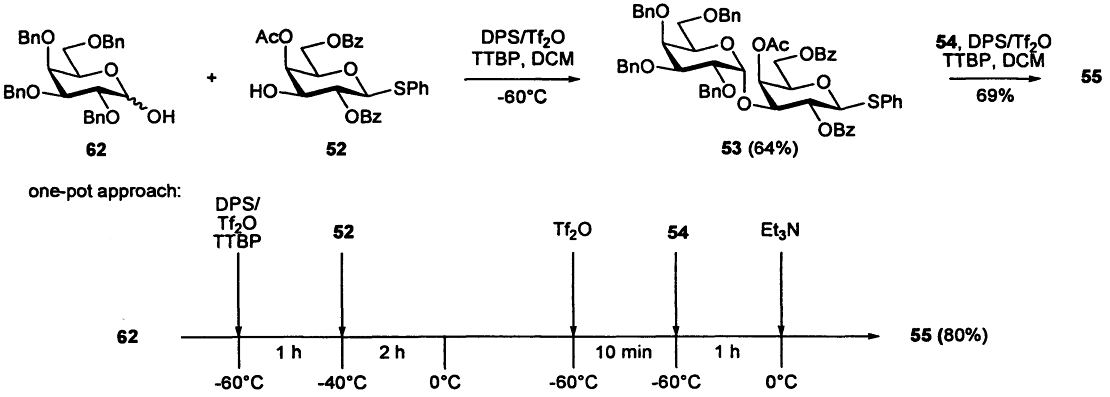
62
+
52
DPS/Tf$_2$O
TTBP, DCM
-60°C
53 (64%)
54, DPS/Tf$_2$O
TTBP, DCM
69%
55
one-pot approach:
DPS/
Tf$_2$O
TTBP
52
Tf$_2$O
54
Et$_3$N
62
1 h
2 h
10 min
1 h
-60°C
-40°C
0°C
-60°C
-60°C
0°C
55 (80%)

Scheme 11

To broaden the scope of the sequential glycosylation strategy, it was investigated whether the assembly of fully protected α–Gal epitope **55** could also be attained in a one-pot procedure. Treatment of 1-hydroxyl donor **62** with DPS/Tf_2O, followed by the addition of acceptor **52** led to the formation of dimer **53** and the regeneration of DPS. The addition of Tf_2O to this reaction mixture led via the formation of the sulfonium species to the activation of the thioglycoside dimer **53** and subsequent condensation with terminal glucosamine **54** gave trisaccharide **55** in 80% yield.

In summary, thioglycosides remain versatile building blocks in the assembly of oligosaccharides. Their application is increased by the development of the here presented glycosylation strategies, using sulfonium ion activator systems.

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Chapter 13

New Aspects of Glycoside Bond Formation: Solid-Phase Oligosaccharide Synthesis

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For solid phase oligosaccharide synthesis various linker types and solid supports were investigated in our group. Finally an ester based methodology with Merrifield resin as solid support was successfully installed. This method employs a hydroxymethylbenzyl benzoate spacer-linker system which is connected to the Merrifield resin. Glycosylation is performed with *O*-glycosyl trichloroacetimidates as powerful glycosylating agents permitting chain extension, branching, and chain termination with the help of *O*-benzyl, *O*-benzoyl, and *N*-dimethylmaleoyl as permanent and *O*-fluorenylmethoxycarbonyl and *O*-phenoxyacetyl as temporary protecting groups. The required steps on solid phase are (i) glycosylation under TMSOTf catalysis, (ii) chemoselective cleavage of temporary protecting groups to generate acceptors, and (iii) product cleavage from the resin with sodium methanolate in methanol following product isolation. This way, various oligosaccharides including a small library of complex-type *N*-glycans was accessible in high overall yields and high purity. Preliminary studies on automated oligosaccharides synthesis were also successful.

In Frontiers in Modern Carbohydrate Chemistry; Demchenko, A.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2007.

Introduction

Oligosaccharides play an important role in various biological processes; therefore the general interest in these compounds, particularly as constituents of glycoconjugates has greatly increased in recent years (*1-4*). As a consequence, oligosaccharide synthesis has become an important issue (*5-10*). Recently, successful solid-phase oligosaccharide syntheses (SPOS) have been developed by several research groups (*11-22*), which exhibit the inherent advantages over solution phase synthesis, such as (i) higher reaction yields due to the use of excess building blocks and/or reagents, (ii) shorter times for the completion of the syntheses, and (iii) convenient purification particularly of intermediates. Also methods to avoid the accumulation of undesired byproducts together with the target molecule have been introduced (*23-26*). However, overall results of isolated pure products have not yet reached those of peptide and nucleotide synthesis on polymer support, therefore no generally accepted strategy has yet appeared for the efficient construction of various complex oligosaccharides on polymer supports. Hence, the development of the technology for automated SPOS is still in progress (*12, 24, 27, 28*).

In this overview different approaches to SPOS pursued in our laboratory are discussed. Finally, these investigations led to a novel methodology which – because of its ease and efficiency – should find general acceptance.

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General Aspects of SPOS

The key issues in SPOS are: (i) selection of the polymer support, (ii) linker design, (iii) choice of the glycosyl donor, (iv) selection of a protecting group pattern for permanent and temporary protection, thus allowing for controlled chain extension and branching, (v) monitoring of the reaction course, and finally (vi) product cleavage from the resin and product characterization.

As solid supports, mainly Merrifield resins having hydroxymethyl, chloromethyl or carboxyl functional groups attached to phenyl rings were selected. These solid supports furnished up to now the best glycosylation results although the dependence on solvent swelling limits the solvent choice. Particularly popular for the solid-phase synthesis of oligonucleotides is controlled-pore glass (CPG) as support (*29*); however, it has not attracted much interest for the synthesis of oligosaccharides (*30*). Its compatibility with polar solvents and the absence of limitations due to solvent swelling requirements should be an advantage; however, this is not yet verified. An important achievement in the use of CPG support for SPOS using *O*-glycosyl trichloroacetimidates as glycosyl donors has been reached by us (*31*). MALDI-TOF and TLC analyses were performed from the cleavage of small support samples to monitor the reactions. This way the synthesis of an α(1-2)-linked trimannoside was achieved; however, overall yields of similar reactions on Merrifield resin proved to be higher. Also soluble polyethylene glycol (PEG) based SPOS (*20, 32*) did not reveal any advantages in our hands (*33*).

The availability of a high yielding and stereoselective glycosylation strategy is of fundamental importance for SPOS. Of the various glycosyl donors employed for this purpose (*34*), *O*-glycosyl trichloroacetimidates are particularly suitable (*5*) (Scheme 1) for the following reason:

- convenient preparation;
- activation by catalytic amounts of acid/Lewis acid;
- no acidity/basicity variation during the reaction course;
- activation compatible with all common protecting groups;
- high glycosyl donor properties;
- α- or β-selectivity generally high (control: solvent, temperature, anchimeric assistance, etc.);
- no formation of water or highly reactive intermediates during the reaction course;
- leaving group inert (nonbasic/nonacidic), stable, readily separable;
- activation orthogonal to various linker types;
- available linker cleavage conditions broad: base and mild acid treatment, hydrogenolysis, metathesis reaction, oxidation, ozonolysis, etc.

The glycosyl donor selection has an important impact on 'permanent' and 'temporary' protecting group and linker selection (Scheme 1: R^2, R^3, R^4, R^6, and L). The permanent protecting groups (cleavage after completion of the oligosaccharide synthesis) have to be orthogonal to the glycosylation conditions and to the cleavage conditions of the temporary protecting groups. Generally, benzyl is selected for hydroxy groups and phthaloyl (Phth), dimethylmaleoyl (DMM) or azido for amino groups. Temporary protecting groups have to be orthogonal to the glycosylation conditions as well. However, the transformation of a glycosyl donor residue after the glycosylation step into an acceptor for further chain extension and/or branching requires one or two orthogonal temporary protecting groups at the glycosyl donor (i.e. **e**-type donors for chain *e*xtension and **b**-type donors for *b*ranching have to be employed, otherwise the chain will be *t*erminated → **t**-type donors). The temporary protecting groups should be removable best under very mild conditions.

The linker has to be regarded as an equivalent of a temporary protecting group as well, thus exhibiting that the linker design strongly influences the selection of the temporary protecting groups. The selection of the highly reactive *O*-glycosyl trichloroacetimidates as glycosylating agents (see above) permits the use of a wide variety of readily cleavable functional groups as linkers, which only need to tolerate mild acid treatment. Thus, other common leaving groups employed in glycosylation reactions, as for instance thioglycosides and pentenyl-type glycosides seem to be particularly suitable as linkers because they

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require a thiophilic or a typical carbon-electrophilic reagent, respectively, for activation and cleavage. Pentenyl-type glycosides or related CC-double bond containing groups permit also cleavage by cross metathesis and/or ring-closing metathesis (RCM). Also sterically demanding *O*-silyl groups and almost all ester groups tolerate the *O*-glycosyl trichloroacetimidate glycosylation conditions, thus permitting their use as linkers in SPOS as well.

Obviously, a particularly wide variety of linkers should be available for SPOS when *O*-glycosyl trichloroacetimidates are employed as glycosyl donors. However, most surprisingly in our work on SPOS the most difficult task was to find a suitable linker which provides the desirable stability during construction of the oligosaccharide on the solid support and the desirable reliable and highyielding product cleavage from the solid support. Therefore, this search for an ideal linker system is employed as subdivision of the following discussions.

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Thioglycosides as Linkers

The first application of *O*-glycosyl trichloroacetimidates as glycosyl donors for the generation of oligosaccharides attached to Merrifield resin was reported by our group (*35*). This approach was based on the use of a thiol functionalized resin (Scheme 2). Among the different supports investigated, resins **1** and **2** shown in Scheme 2, simply derived from Merrifield resin having chloromethyl groups by *O*- or *S*-alkylation, proved to be the most efficient in terms of reaction yields and chemical stability. These two resins turned out to be very good glycosyl acceptors, thus allowing very efficient introduction of the first sugar residue. This method was successfully employed in the synthesis of (1-6)-linked oligomers of glucose (*35*), α(1-2)-linked oligomers of mannose (*18*), Fucα(1-2)Man disaccharide (*18*) and in the preparation of a pentasaccharide related to *N*-glycan chains (*17*) (see Schemes 3 and 4).

N-Glycosylation is a well-known feature of many natural proteins in eukaryotic organisms that contributes to their structural as well as biological properties (*1, 2*). These naturally occurring *N*-glycans display a high degree of structural diversity, and an array of different carbohydrate isoforms are synthesized even on one type of protein. Varied glycan patterns are expressed in different types of tissues at different stages of embryonic development. Consequently, for the chemical synthesis, particularly of the complex type *N*-glycans (see Scheme 3), an approach is needed that intrinsically permits a high degree of variation, hence a combinatorial approach based on SPOS seemed to be particularly suitable. Because one of the most difficult tasks constitutes the chemical synthesis of complex-type *N*-glycans (*36*), consisting of a branched core pentasaccharide having different antennae-like side chains, this task seemed to be particularly appropriate to demonstrate the power of SPOS.

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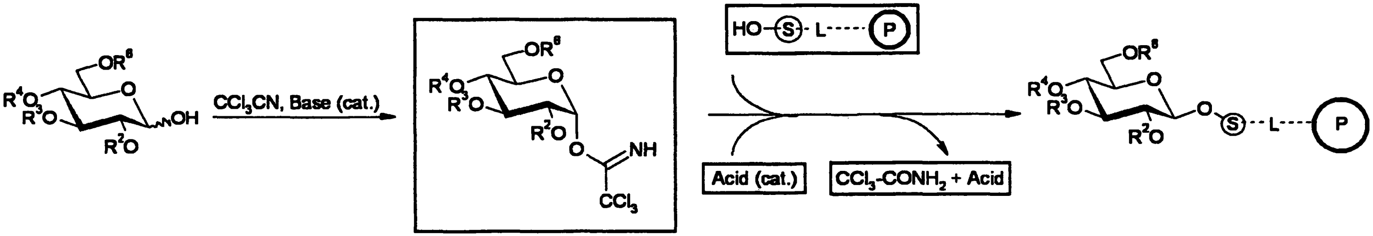
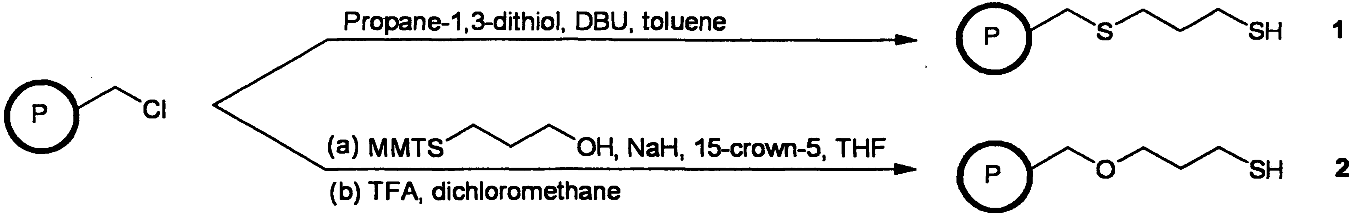

OR⁶
R⁴O
R³O
R²O
OH
CCl₃CN, Base (cat.)
NH
CCl₃
HO-S-L-----P
Acid (cat.)
CCl₃-CONH₂ + Acid
S
L
P

Scheme 1. SPOS – O-Glycosyl trichloroacetimidates as glycosyl donors. S = Saccharide, L = Linker, P = Polymer support.


P
Cl
Propane-1,3-dithiol, DBU, toluene
(a) MMTS OH, NaH, 15-crown-5, THF
(b) TFA, dichloromethane
S
SH
1
O
SH
2

Scheme 2. Thiol functionalization of resins for oligosaccharide synthesis.

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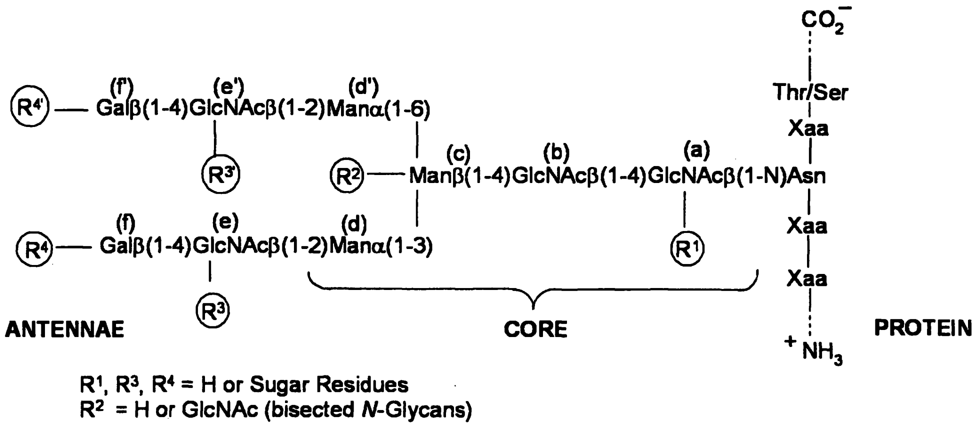
R4'
(f')
Galβ(1-4)GlcNAcβ(1-2)Manα(1-6)
(e')
(d')
R3'
R2
(c)
(b)
(a)
Manβ(1-4)GlcNAcβ(1-4)GlcNAcβ(1-N)Asn
R4
(f)
(e)
(d)
Galβ(1-4)GlcNAcβ(1-2)Manα(1-3)
R3
R1
CO2−
Thr/Ser
Xaa
Xaa
Xaa
+NH3
ANTENNAE
CORE
PROTEIN
R1, R3, R4 = H or Sugar Residues
R2 = H or GlcNAc (bisected N-Glycans)

Scheme 3. Typical oligosaccharide structures found as complex-type N-*glycans. Retrosynthetic analysis → disconnections 1-4.*

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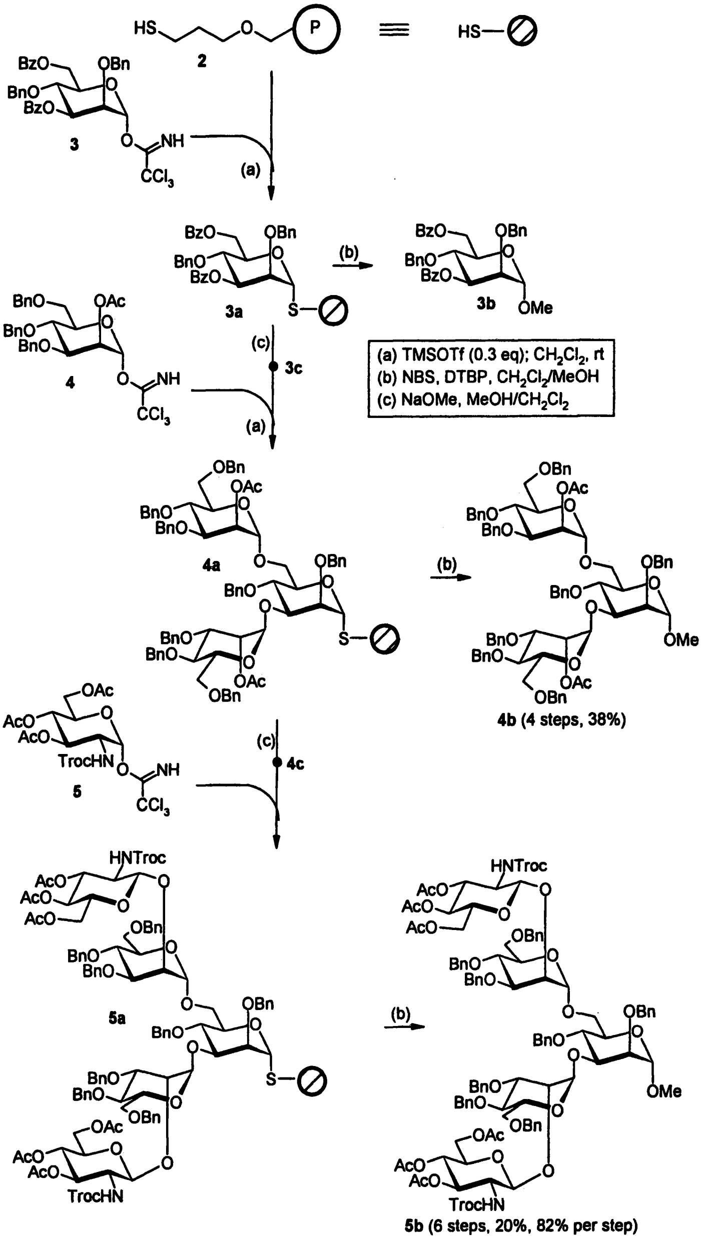
HS
O
P
2
≡
HS
BzO
OBn
BnO
BzO
O
3
O
NH
CCl3
(a)
3a
S
(b)
3b
OMe
BnO
OAc
4
(c)
3c
(a) TMSOTf (0.3 eq); CH2Cl2, rt
(b) NBS, DTBP, CH2Cl2/MeOH
(c) NaOMe, MeOH/CH2Cl2
4a
4b (4 steps, 38%)
AcO
TrocHN
5
4c
HNTroc
5a
5b (6 steps, 20%, 82% per step)

Scheme 4. Thiol-linker based SPOS of a complex-type N-*glycan fragment.*

The SPOS of an important pentasaccharide moiety of *N*-glycans (Scheme 4) started from thiol resin **2**. The first glycosylation using mannosyl donor **3** furnished resin **3a** and cleavage led to product **3b**. After *O*-debenzoylation, acceptor resin **3c** was used as starting material for a glycosylation (with donor **4**) – deprotection – glycosylation (with donor **5**) sequence in which two glycosidic bonds (→ **4a**, **5a**) were formed during each glycosylation step. The target pentasaccharide **5b** was obtained from **5a** in 20% overall yield after six steps (82% per step). The strategy used for this work was based on anchimeric assistance to ensure stereoselective 1,2-*trans*-glycosylation by employing previously described donors. The target pentasaccharide **5b** was one of the most complex oligosaccharides synthesized on a solid support by then. However, careful investigation of the reactions (particularly the glycosylation reactions) exhibited that thioglycosides are not as stable to the solid phase reaction conditions as anticipated from literature reports: material was lost from the resin which could be detected in the solvents used for washing. Therefore it was decided to employ a hopefully better linker system.

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Pentenyl and Allyl-type Linkers

Pentenyl glycosides have been successfully employed as glycosyl donors based on their activation with typical carbon electrophiles (*37*). Because of their stability towards mild acids, pentenyl-type intermediates as shown in Scheme 5 seemed to be useful linker systems in SPOS with *O*-glycosyl trichloroacetimidates as donors.

Readily available Merrifield resin **7** (as one example) having a pentenyl linker, as shown in Scheme 6, was glycosylated with lactosyl donor **6** to afford the desired lactosyl resin **8**. Yet, cleavage investigations under standard conditions (excess *N*-iodosuccinimide, catalytic amounts of TMSOTf in the presence of methanol) (*38*) furnished only minor amounts of the desired methyl lactoside **9** (< 5%); mainly cleavage product **10** was obtained. However, the total product yield (**9** + **10** < 50%) was far from satisfactory and the results could not be improved by varying the cleavage conditions and by using structurally varied pentenyl-type linkers. Therefore, it was decided to investigate this type of compounds in cross-metathesis and ring-closing metathesis (RCM) based cleavage reactions; the structural requirements for those reactions should be also compatible with the use of *O*-glycosyl trichloroacetimidate glycosylations.

In our hands RCM proved to be more successful than cross-metathesis (*24*, *39*); therefore, some RCM results will be discussed (*40*, *41*). It was particularly pleasing that the validity of this approach could be already demonstrated in some preliminary work on oligosaccharide synthesis (*40*). In the course of our ongoing program concerning the SPOS of milk oligosaccharides (*41*) the linker **11** available to RCM was prepared in two steps from Merrifield resin and hydroxymethyl-octadienol (Scheme 7). The loading of this resin was calculated

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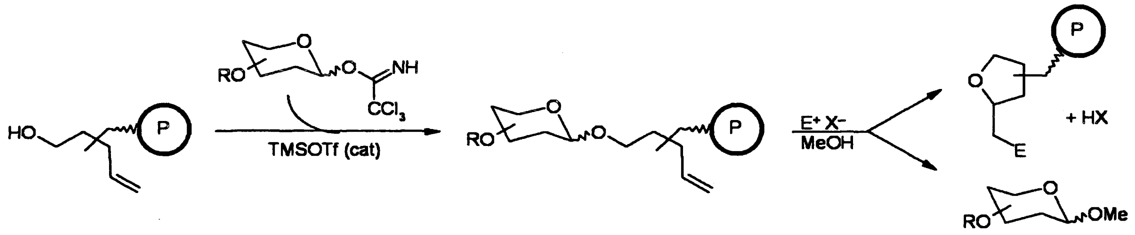
HO
P
RO
O
O
NH
CCl3
TMSOTf (cat)
RO
O
O
P
E+ X-
MeOH
O
P
E
+ HX
RO
O
OMe

Scheme 5. Pentenyl-type linker for SPOS.

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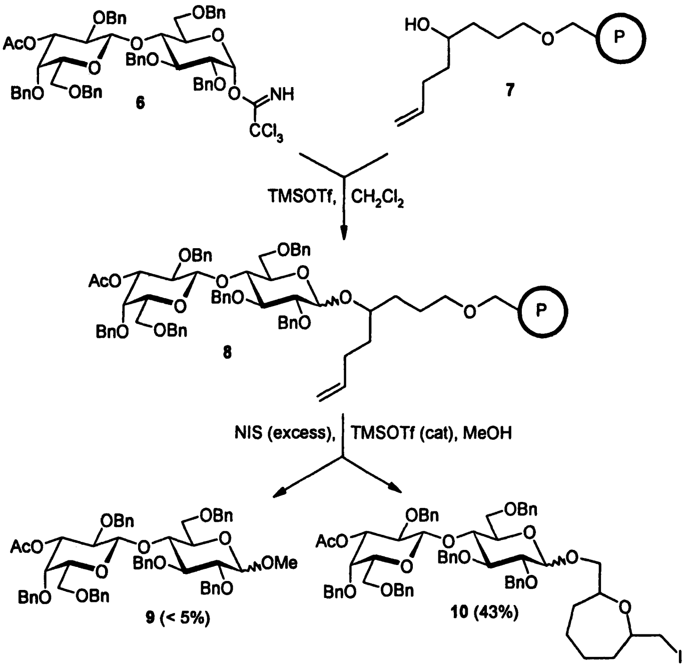

Scheme 6. Investigation towards a pentenyl-type linker in SPOS.

to be 0.22 mmol/g. Application of this resin in the glycosylation with lactosyl donor **12**, permitting branching based on the orthogonality of an *O*-acetyl and an *O*-silyl protecting groups, led in two successive glycosylations with DMM-protected lactosamine donor **14** via **13** and **15** to resin-bound branched lacto-neo-hexaose derivative **16**. Cleavage with the help of Grubb's catalyst afforded target molecule **17** in an encouraging overall yield (13% over 6 steps; 71% per step). Work aimed at extending this approach to the synthesis of other complex oligosaccharides (for instance, the synthesis of Lewis A pentasaccharide) exhibited once in a while unpredictable failures in the cleavage step. Therefore, we decided to proceed to other linker types.

From the analytical point of view, reactions were efficiently monitored using a combination of FTIR (Fourier transform infrared), TLC (thin-layer chromatography), and MALDI-TOF mass spectrometry analysis of crude product mixtures resulting from the cleavage of small resin samples.

Silyl Linker

The compatibility of sterically demanding *O*-silyl protecting groups with *O*-glycosyl trichloroacetimidate based glycosylations was reason to investigate

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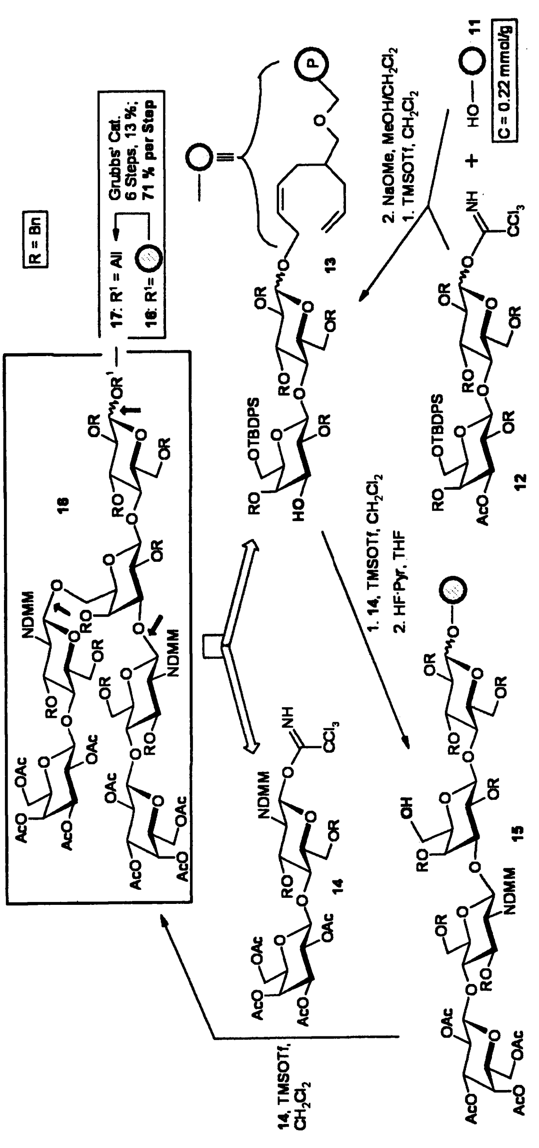
R = Bn
17: R¹ = All
16: R¹ =
Grubbs' Cat.
6 Steps, 13 %;
71 % per Step
18
13
14
15
12
11
14, TMSOTf, CH₂Cl₂
1. 14, TMSOTf, CH₂Cl₂
2. HF·Pyr, THF
2. NaOMe, MeOH/CH₂Cl₂
1. TMSOTf, CH₂Cl₂
C = 0.22 mmol/g

*Scheme 7. SPOS of branched lacto-*N-neo-*hexaose with the help of a linker accessible to RCM based cleavage.*

structurally related silyl groups as linkers in SPOS (*42*). They were already successfully employed in glycosylations with the help of epoxides (*43*). To this end, we started from 4-bromocinnamate and transformed it into linker-loaded resin **18** as shown in Scheme 8 (*42*). One prominent target molecule, the synthesis of which was probed with this methodology, was the Galili antigen Galα(1-3)Galβ(1-4)GlcNAc (*44*). An important finding in connection with this work was the compatibility of hydroxy group protection by fluorenylmethyloxycarbonyl (Fmoc) as temporary *O*-protecting group with *O*-glycosyl trichloroacetimidate formation, thus permitting a convenient base-catalyzed liberation of hydroxy groups after the glycosylation step. In addition, it was found that *O*-phenoxyacetyl (PA) groups can be quantitatively removed with mild nucleophiles even in the presence of *O*-benzoyl and *O*-acetyl groups. Hence, glycosylation of silyl linker **18** with 4-*O*-Fmoc containing glucosamine donor **19** and then Fmoc cleavage wit triethylamine afforded resin bound acceptor **20**. Following glycosylation with 3-*O*-PA containing galactosyl donor **21** led to disaccharide carrying resin **22**. Cleavage of this disaccharide from the resin with tetrabutylammonium fluoride (TBAF) in HOAc/THF furnished lactosamine derivative **23** in 70% yield, corresponding to 93% yield per step. Glycosylation of resin **22** with galactosyl donor **24** for α-linkage formation and chain termination afforded resin bound target molecule **25**. The trisaccharide was cleaved from the resin as described above to afford the *O*-benzyl and *N*-phthaloyl protected target molecule **26**, carrying the desired anomeric hydroxypropyl residue for affinity chromatography purposes, in 38% yield after seven steps (→ 87% per step). This way, for the first time in a complex SPOS a very good overall result could be obtained. Therefore, the general applicability of this methodology seemed to be guaranteed.

Inspection of these results exhibited that even a simpler and more efficient methodology should be available. The stability of *O*-benzoyl groups to the cleavage conditions of the temporary Fmoc and PA groups led to the conclusion that SPOS can be totally based on ester groups for the linker and for the required temporary protecting groups permitting chain extension and branching. This way, the valuable *O*-silyl group would be available for other protecting group manipulations.

Benzoate Esters as Linker

Obviously, the commercial availability of Merrifield resin bearing benzoic acid functions for immediate benzoate ester linkage formation is a great advantage in the ester based approach to SPOS. Thus, carbohydrates can be directly linked to the resin via one of the hydroxy groups of the sugar residue (direct linkage to the anomeric hydroxy groups is not recommended because of its higher reactivity) or they can be linked via a diol spacer to the anomeric position. Both methods have been investigated and will be discussed.

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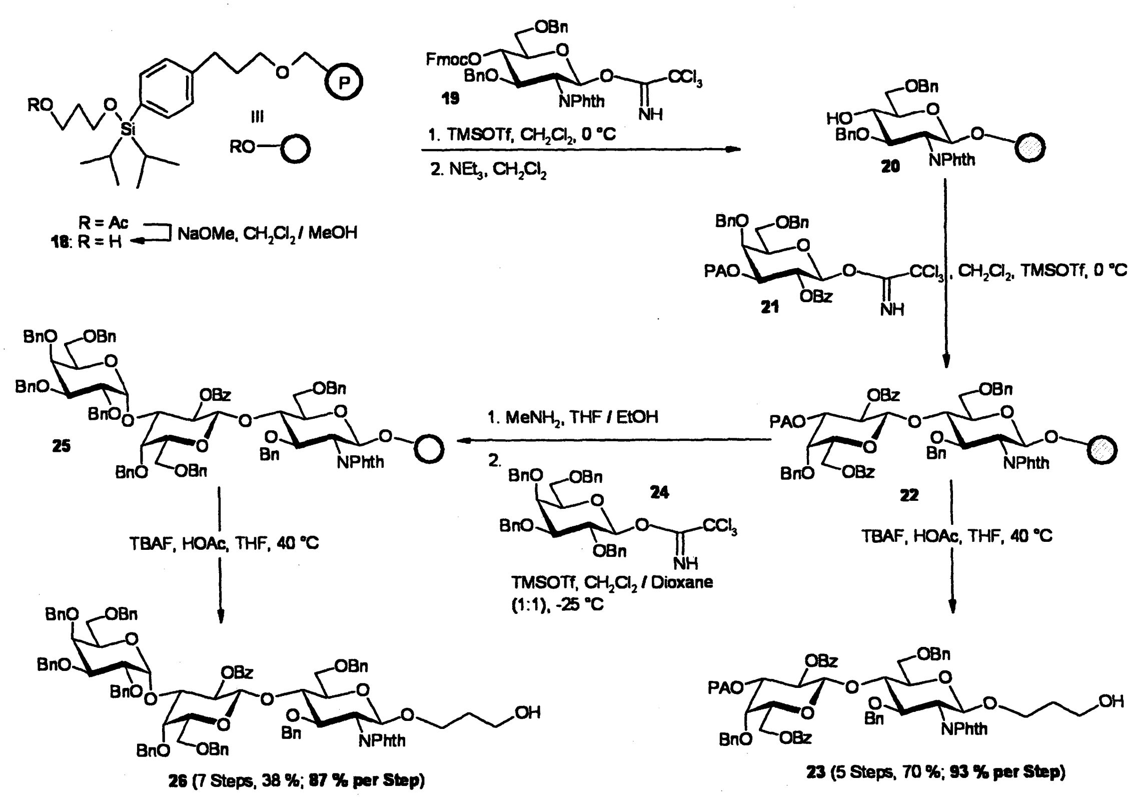
RO
P
III
RO
R = Ac
18: R = H
NaOMe, CH2Cl2 / MeOH
FmocO
BnO
OBn
19
NPhth
CCl3
NH
1. TMSOTf, CH2Cl2, 0 °C
2. NEt3, CH2Cl2
HO
20
21
PAO
OBz
, CH2Cl2, TMSOTf, 0 °C
22
Bn
1. MeNH2, THF / EtOH
2.
24
TMSOTf, CH2Cl2 / Dioxane (1:1), -25 °C
25
TBAF, HOAc, THF, 40 °C
TBAF, HOAc, THF, 40 °C
OH
26 (7 Steps, 38 %; 87 % per Step)
23 (5 Steps, 70 %; 93 % per Step)

Scheme 8. Synthesis of the Galili-antigen.

(a) Linkage of the resin to the primary hydroxy group of the sugar residue

Out of the various examples investigated with this method, the reiterative solid phase cycle for the construction of the biologically important α(1-2)-linked mannoside oligomers (*45*, *46*) (Scheme 9) will be discussed here (*47*). The required mannose building blocks **27** and **28** bearing Fmoc groups were readily obtained. The primary hydroxy group of **27** was attached to the resin to furnish mannoside-linked resin **29**. In this approach loading of the resin was determined to be 0.20 mmol/g by two different methods (*47*). Removal of the Fmoc group by treatment of resin **29** with triethylamine furnished acceptor **30**. The chain distance for a six-membered ring of six bonds between the acceptor hydroxy group and the linkage position in **30** seemed to be sufficient for successful glycosylation with donor **28** furnishing **31**-n (n = 1). Fmoc-deprotection and repetition of the glycosylation (with **28** → **31**-n) and deprotection sequence (→ **32**-n) in a cyclic manner (n = 1 to 5) and transesterification cleavage with methanol in the presence of sodium methanolate furnished the corresponding oligomannosides **33**-n. The target molecules **33-1**, **33-3**, **33-5** were isolated in anomerically pure form in high overall yields and additional elongation at the non-reducing end is available. Obviously, this simple ester-type linker based SPOS is highly efficient, particularly when compared with results obtained via the thioglycoside based linker method. Similar results were obtained in the synthesis of more complex oligosaccharides (*42*, *47*).

Even use of a more reactive phenoxyacetate based linker led to excellent results (*16*). However, these investigations were not extended because of the value of the PA group as temporary protecting group for the synthesis of branched oligosaccharides (see below).

(b) Linkage of the resin with the anomeric hydroxy group via a diol spacer

In an endeavour to further develop the benzoate ester methodology the synthesis of human milk branched lacto-*N*-*neo*-hexaose was resumed (see Schemes 7 and 10) (*13*). An important requirement was the design of a lactose building block possessing orthogonal protecting groups in the 3b- and 6b-*O*-positions. For this branching, lactosyl trichloroacetimidate **35** was selected bearing levulinoyl (Lev) and Fmoc groups. The linker **34** was readily obtained from 4-bromocinnamate and the lactosamine donor **14** was already previously employed (Scheme 7). Resin **34** was glycosylated with 3 equiv of new *O*-lactosyl trichloroacetimidate **35** under trimethylsilyl triflate (TMSOTf) activation to furnish the corresponding support-bound disaccharide **36**. The batch of resin **36** was then split into two pools in order to prepare the target hexasaccharide via two different routes, thus emphasizing the orthogonality between the Fmoc and Lev protecting groups.

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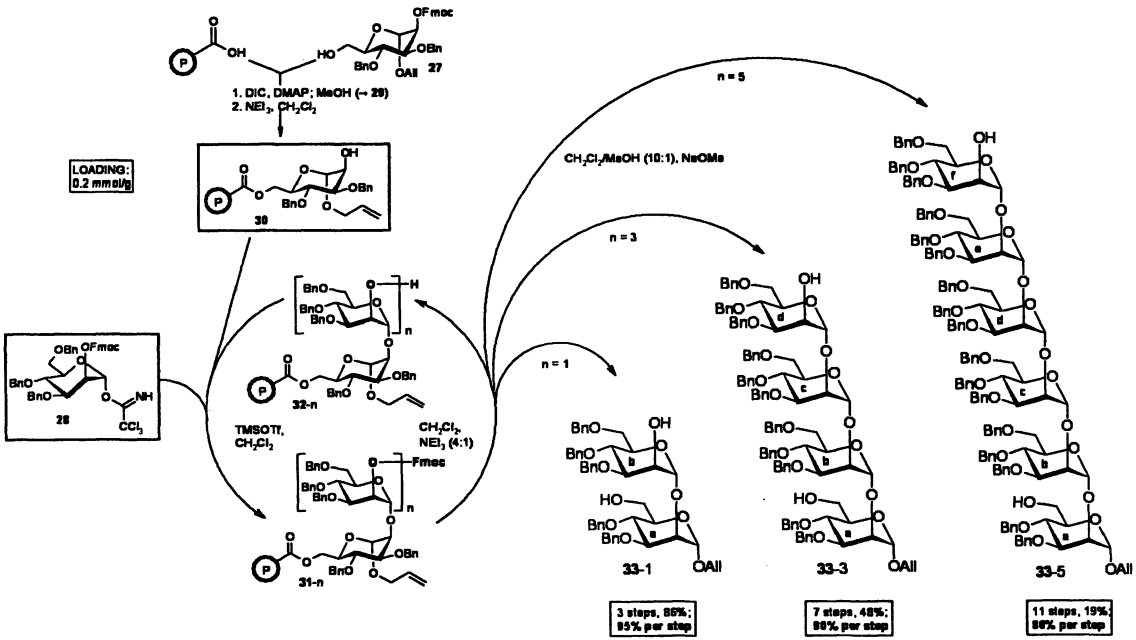
P
OH
HO
OFmoc
OBn
BnO
OAll
27
1. DIC, DMAP; MeOH (→ 29)
2. NEt$_3$, CH$_2$Cl$_2$
LOADING:
0.2 mmol/g
OH
OBn
30
OBn OFmoc
BnO
NH
CCl$_3$
28
H
n
32-n
TMSOTf,
CH$_2$Cl$_2$
CH$_2$Cl$_2$,
NEt$_3$ (4:1)
Fmoc
31-n
n = 1
n = 3
n = 5
CH$_2$Cl$_2$/MeOH (10:1), NaOMe
a
b
c
d
e
f
HO
33-1
33-3
33-5
OAll
3 steps, 86%;
95% per step
7 steps, 48%;
90% per step
11 steps, 19%;
86% per step

Scheme 9. *SPOS of α(1-2)-linked mannan with the help of a benzoate linker to the primary hydroxy group of mannose.*

Concerning the first protocol, the Fmoc group was selectively removed in the presence of the Lev group by treatment of **36** with triethylamine in CH_2Cl_2. No loss of the Lev group was observed during this reaction. Next, an elongation was performed by reaction with lactosamine donor **14** upon activation with TMSOTf, leading to resin-bound tetrasaccharide **37**. The Lev group was subsequently removed by reaction with hydrazinium acetate, releasing the *C*-6b primary hydroxyl group. In order to exhibit further the efficiency of Fmoc-containing *O*-glycosyl trichloroacetimidates for solid-phase oligosaccharide synthesis, the second lactosamine moiety was introduced by using instead of lactosamine donor **14** at first glucosamine donor **38** and then galactosyl donor **39**. Thus, preparation of a small library of branched oligosaccharides starting from resin-bound lactose **36** is feasible. Glycosylation with **38** (4 equiv) and TMSOTf as catalyst and then removal of the Fmoc group furnished resin-bound pentasaccharide as acceptor. Elongation with **39** as donor under TMSOTf activation furnished the corresponding resin-bound hexasaccharide **40**. Cleavage from the polymer support under alkaline conditions (MeONa) was executed twice to give an anomeric mixture (β/α 1:1) of hexasaccharide **41** in 43% overall yield from **34** (90% per step, eight steps).

A second route to the synthesis of **41** was performed in which the Lev group of **36** was first selectively removed in the presence of the Fmoc group using the conditions described above. This time, the elongation with **14** started at the 6b-*O*-position, thus allowing also the isolation of the corresponding immobilized tetrasaccharide. After removal of the Fmoc group furnishing **42**, the branching of the oligosaccharide was continued at the 3b-*O*-position, applying exactly the sequence used during the first route. Thus the desired compound **41** was obtained in almost the same overall yield (42%) as described above. The usefulness of lactose building block **35** and the efficiency of the selected protecting group pattern was demonstrated in the synthesis of **41** with two different approaches.

With derivative **41** in hand, the removal of the protecting groups was undertaken. At first, the two DMM groups were removed and the released amino groups were converted into *N*-acetamido functions by treatment with acetic anhydride. Finally, the benzylic groups were smoothly cleaved by catalytic hydrogenation over palladium to produce, after acetylation, the linker-free peracetylated lacto-*N*-*neo*-hexaose **43**.

The novel benzoic acid ester type linker has proven to be inert to all reaction conditions all along the construction of the oligosaccharide. This linker offers a great advantage of releasing a benzylic aglycone moiety which can be easily removed by hydrogenolysis. Thus, the preparation of fully unprotected oligosaccharides can be readily carried out.

With these excellent results in hand the SPOS of branched complex-type *N*-glycans was also reinvestigated (*34*). For this purpose, commercially available benzene-1,4-dimethanol was selected as spacer and linker to the benzoyl residue, thus leading to readily available linker loaded resin **44** (Scheme 11).

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With this system and the building blocks **19**, **28**, and **45** shown in the scheme up to a branched hexasaccharide *N*-glycan was successfully obtained (11 steps on solid phase, 19% overall yield; 86% per step) (see *34*). This was a major improvement over the results reported in Scheme 4. A careful study of this reaction sequence revealed that the second glycosylation step is incomplete, thus leading also to byproduct formation (*48*). Therefore, a capping step of unreacted hydroxy groups based on benzoyl isocyanate reaction was introduced (*25*). As expected and shown in Scheme 11, this way clean product formation could be gained, however the overall yield was not increased.

Hence, it was decided to increase the reactivity of the first sugar residue by replacing the phthalimido group by the azido group which showed the desired effect (*49*). However, introduction of glucosamine residues at the nonreducing end would be hampered because the stereocontrol with the help of the nitrile effect (*50*) was less pronounced on solid support than in solution (*48*).

Further inspection of the complex-type *N*-glycan synthesis revealed that the reactivity decrease of *N*-phthaloyl protected acceptors next to the Merrifield resin should be due to unfavourable interaction of the flat phthaloyl residues with the phenyl rings of the Merrifield resin, this way limiting the access to the 4-hydroxy group. (*27*). Simple replacement of the phthaloyl with the DMM group for amino protection solved this problem (*27*). Therefore, the strategy outlined in Scheme 12 was followed for the SPOS of complex-type *N*-glycans: The ester based methodology comprises (i) different types of esters: as linker and for chain termination benzoate groups and as temporary protecting groups for chain extension and branching Fmoc and PA which could be chemoselectively cleaved (in the sequence Fmoc and then PA); (ii) benzyl for permanent *O*-protection and for the spacer to the anomeric centre at the reducing end sugar, thus providing after final product cleavage from the resin a structurally defined target molecule, (iii) *O*-glycosyl trichloroacetimidates of type **e**, **b**, or **t** (for chain extension, branching or termination) as powerful glycosyl donors, which can be readily activated by catalytic amounts of (Lewis) acid, and (iv) benzoic acid residues containing Merrifield resin for the linkage of the hydroxymethylbenzyl spacer. Hence, retrosynthesis of a typical *N*-glycan molecule containing the core pentasaccharide and some antennae leads to spacer-linker connected Merrifield resin **44** and to glycosyl donors **19**, **28**, **46-50** which can be selectively converted into acceptors on resin (**e** and **b**-type donor building blocks). Thus, as indicated on Scheme 12, for successful SPOS only four simple procedures are required: (a) glycosidation under TMSOTf catalysis, (b) product cleavage under transesterification conditions, (c) selective Fmoc cleavage under basic conditions, and (d) selective PA cleavage under milder transesterification conditions.

Application of this methodology to the synthesis of linear and branched α-connected mannosides and of LacNAcβ(1-2)-mannosides led to yields of 93-97% per step.

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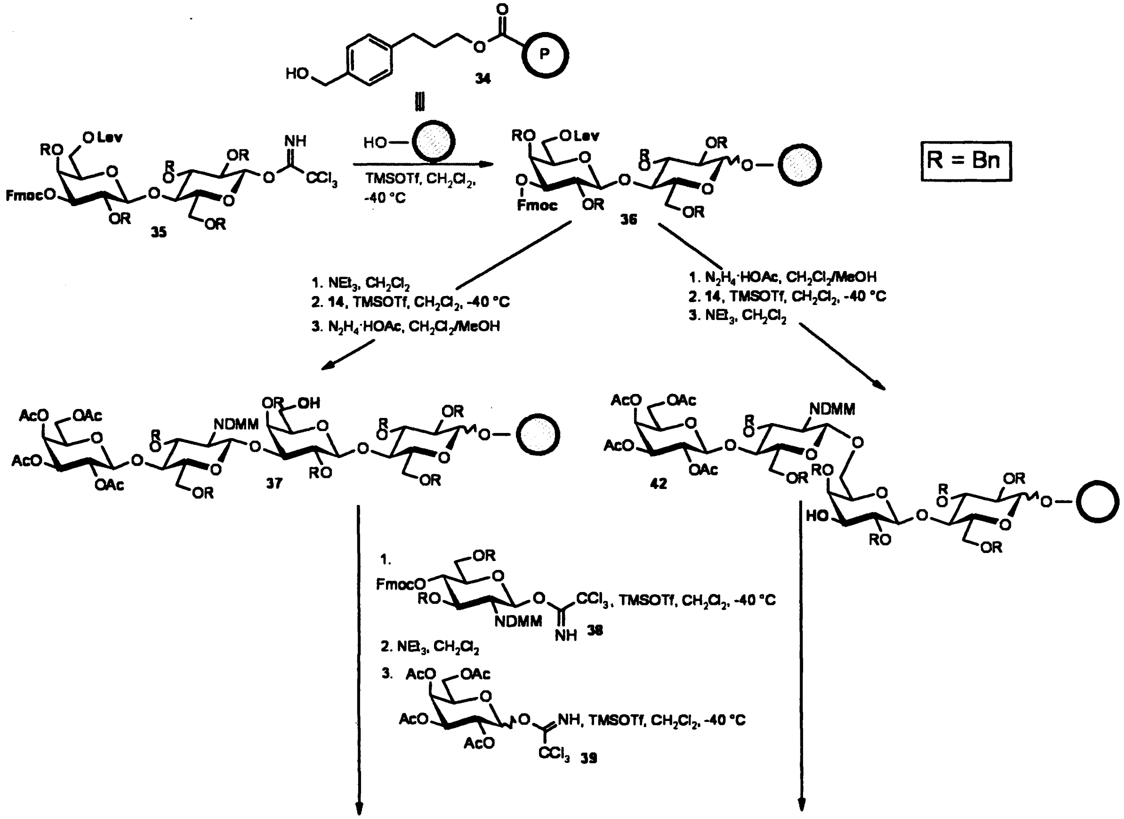
34
35
TMSOTf, CH$_2$Cl$_2$, -40 °C
36
R = Bn
1. NEt$_3$, CH$_2$Cl$_2$
2. 14, TMSOTf, CH$_2$Cl$_2$, -40 °C
3. N$_2$H$_4$·HOAc, CH$_2$Cl$_2$/MeOH
1. N$_2$H$_4$·HOAc, CH$_2$Cl$_2$/MeOH
2. 14, TMSOTf, CH$_2$Cl$_2$, -40 °C
3. NEt$_3$, CH$_2$Cl$_2$
37
42
1.
CCl$_3$, TMSOTf, CH$_2$Cl$_2$, -40 °C
38
2. NEt$_3$, CH$_2$Cl$_2$
3.
NH, TMSOTf, CH$_2$Cl$_2$, -40 °C
39

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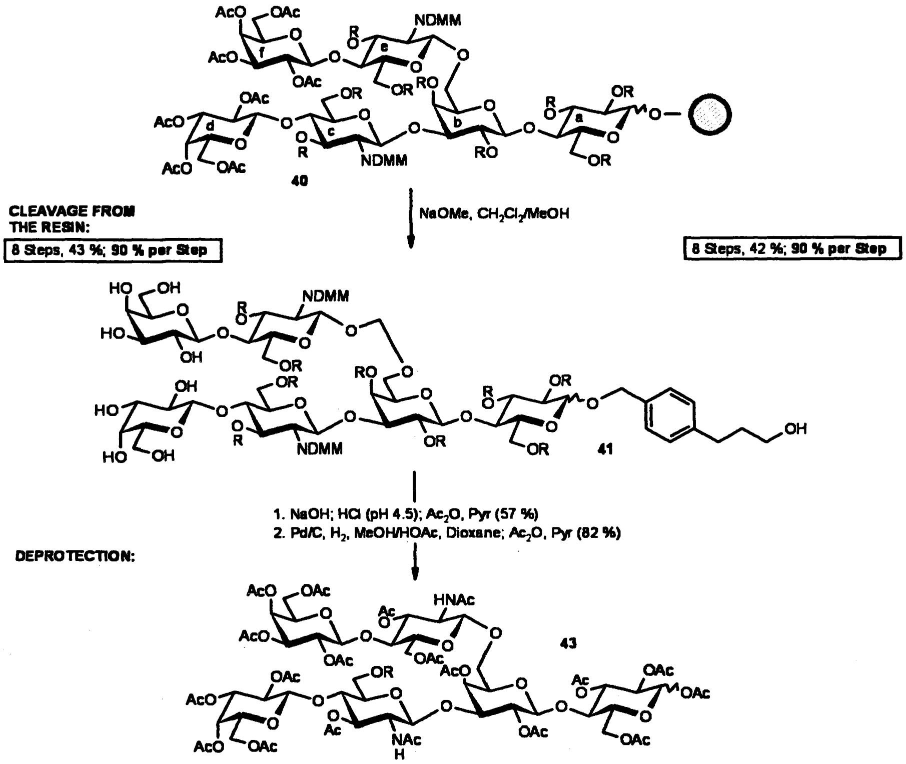
40
NaOMe, CH_2Cl_2/MeOH
CLEAVAGE FROM THE RESIN:
8 Steps, 43 %; 90 % per Step
8 Steps, 42 %; 90 % per Step
41
1. NaOH; HCl (pH 4.5); Ac_2O, Pyr (57 %)
2. Pd/C, H_2, MeOH/HOAc, Dioxane; Ac_2O, Pyr (82 %)
DEPROTECTION:
43

*Scheme 10. Synthesis of lacto-*N-neo-*hexaose with the help of a benzoate linker.*

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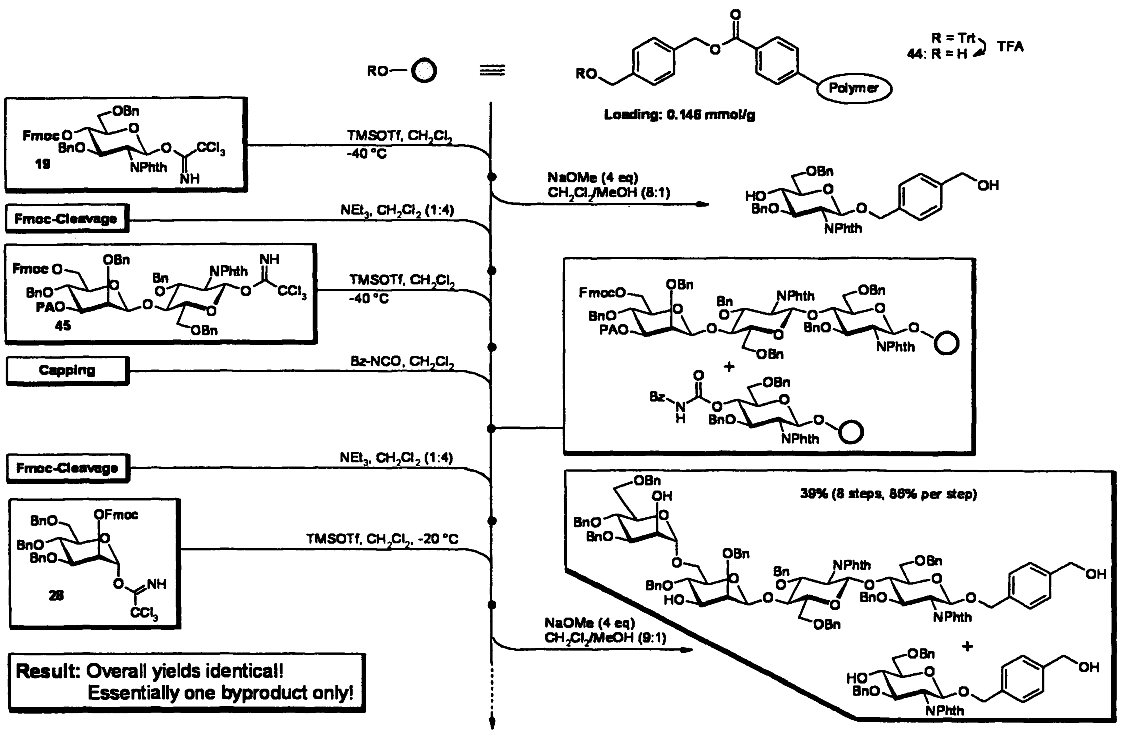
RO—○ ≡
RO
Polymer
R = Trt
44: R = H
TFA
Loading: 0.146 mmol/g
19
Fmoc-Cleavage
45
Capping
Fmoc-Cleavage
28
TMSOTf, CH₂Cl₂ -40 °C
NEt₃, CH₂Cl₂ (1:4)
TMSOTf, CH₂Cl₂ -40 °C
Bz-NCO, CH₂Cl₂
NEt₃, CH₂Cl₂ (1:4)
TMSOTf, CH₂Cl₂, -20 °C
NaOMe (4 eq) CH₂Cl₂/MeOH (8:1)
NaOMe (4 eq) CH₂Cl₂/MeOH (9:1)
39% (8 steps, 88% per step)
Result: Overall yields identical!
Essentially one byproduct only!

Scheme 11. SPOS of N*-glycans employing BzNCO capping.*

With these excellent results in hand, the synthesis of the *N*-glycan core structure and of branched *N*-glycans was undertaken (Scheme 13). To this end, **44** was first glycosylated with *N*-DMM protected glucosamine donor **38** to afford sugar connected resin which after resin cleavage and *O*-acetylation afforded β-linked glycoside **51** in high yield. Fmoc cleavage from this resin and again glycosylation with **38** and then product cleavage from the resin gave chitobioside **52** in 65% overall yield. Reaction of the monoglycosylated resin with disaccharide donor **47** furnished resin bound trisaccharide again in very good yield, as demonstrated by product cleavage from the resin and per-*O*-acetylation affording trisaccharide **53**. After Fmoc cleavage leading to **53P-F** this resin was used in two directions. Mannosylation with chain terminating mannosyl donor **48** led to the desired glycosylated resin as proven by product **54**. PA-cleavage and again mannosylation with **48** furnished in the usual manner *N*-glycan pentasaccharide core structure **55** after nine steps in 39% yield (90% per step).

For further chain extension and branching **53P-F** was glycosylated with mannosyl donor **28** which gave after cleavage of the product from the resin and per-*O*-acetylation also **54** in practically the same yield as obtained before. Fmoc cleavage and then glycosylation with chain terminating glucosamine donor **46** led in the usual manner to pentasaccharide **56**. Selective cleavage of the PA group and then glycosylation with mannosyl donor **28** furnished *N*-DMM protected branched hexasaccharide **57** which was obtained after 11 steps in 30% overall yield (90% per step). The previously reported SPOS of an *N*-Phth protected analogue of **57** was obtained in only 19% overall yield, thus exhibiting the reactivity difference between the *N*-phthaloyl and the *N*-DMM protecting groups. Reaction of **53P-F** with mannosyl donor **28**, Fmoc cleavage and then reaction with glucosamine donor **38** permitting chain extension, gave after cleavage of the product from the resin and per-*O*-acetylation also **56** in practically the same yield as reported above. Fmoc cleavage from this resin and then galactosylation with chain terminating galactosyl donor **50** provided after standard cleavage conditions linear hexasaccharide **58**. Phenoxyacetyl group removal from the resin and then mannosylation with donor **28** led to resin bound heptasaccharide and finally, after 13 steps on the resin, to branched target molecule **59** in 22% overall isolated yield (89% per step). All acyloxymethylbenzyl glycosides obtained after cleavage of the product from the resin and per-*O*-acetylation (i.e. compounds **51-59**), required for purification essentially only one chromatographic step by flash chromatography or medium pressure chromatography which exhibits the efficiency and versatility of this straight forward approach to solid phase oligosaccharide synthesis. The structural assignments are based on NMR and MS data.

The developed highly successful SPOS methodology based on a benzoate linker is summarized in Scheme 14. It is technically simple, thus lending itself available to automation, which was successful with an accordingly designed

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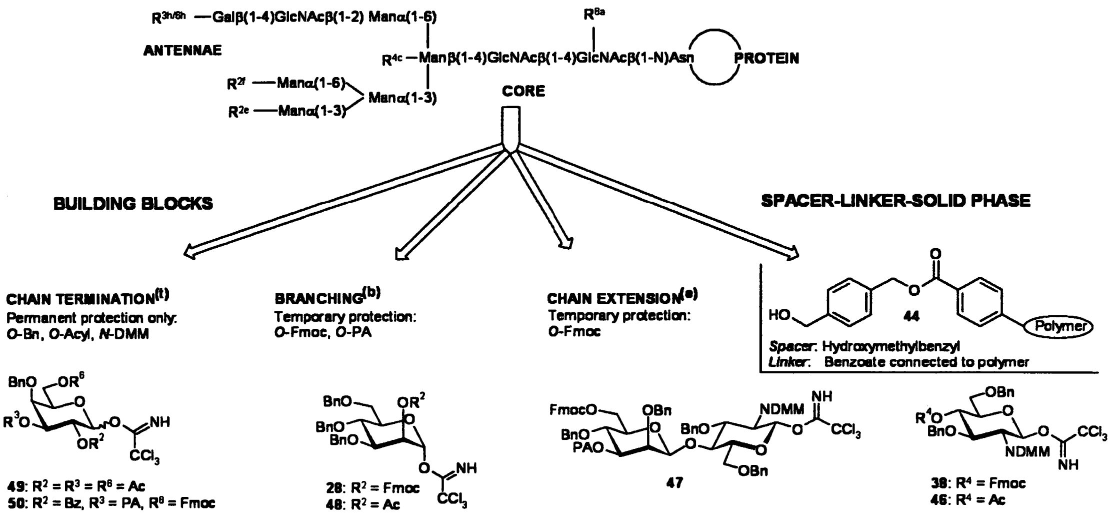
R3h/6h —Galβ(1-4)GlcNAcβ(1-2) Manα(1-6)
ANTENNAE
R2f —Manα(1-6)
R2e —Manα(1-3)
Manα(1-3)
R4c —Manβ(1-4)GlcNAcβ(1-4)GlcNAcβ(1-N)Asn
R8a
PROTEIN
CORE
BUILDING BLOCKS
SPACER-LINKER-SOLID PHASE
CHAIN TERMINATION(f)
Permanent protection only:
O-Bn, O-Acyl, N-DMM
BRANCHING(b)
Temporary protection:
O-Fmoc, O-PA
CHAIN EXTENSION(e)
Temporary protection:
O-Fmoc
HO
O
44
Polymer
Spacer: Hydroxymethylbenzyl
Linker: Benzoate connected to polymer
BnO
OR6
R3O
OR2
NH
CCl3
49: R2 = R3 = R6 = Ac
50: R2 = Bz, R3 = PA, R6 = Fmoc
BnO
BnO
BnO
OR2
NH
CCl3
28: R2 = Fmoc
48: R2 = Ac
FmocO
OBn
BnO
PAO
BnO
NDMM
NH
CCl3
OBn
47
R4O
BnO
OBn
NDMM
CCl3
NH
38: R4 = Fmoc
46: R4 = Ac

REACTIONS ON SOLID PHASE

(a) **Glycosidation**: O-Glycosyl Trichloroacetimidates, TMSOTf (cat.), CH_2Cl_2, 0 to -40 °C
(b) **Product cleavage**: NaOMe (4 eq), CH_2Cl_2/MeOH (8:1); Ac_2O, Pyr
(c) **Fmoc cleavage**: NEt_3/CH_2Cl_2 (1:6)
(d) **PA cleavage**: NaOMe (0.5 eq), CH_2Cl_2/MeOH (8:1)
} UV-Monitoring possible

Scheme 12. SPOS of N*-glycans based on a hydroxymethylbenzyl benzoate spacer-linker and DMM as amino protecting group.*

synthesizer (*28*). This way further process and hence yield optimisation will be possible, thus adding to the overall power of this highly efficient methodology for SPOS. The very positive characteristics, as outlined her, should make this method attractive for general acceptance.

Conclusion

The results obtained on SPOS can be compiled in the following statements:

(1) *O*-Glycosyl trichloroacetimidates are ideal glycosyl donors for SPOS because they combine high glycosyl donor properties with catalytic activation, thus avoiding the accumulation of avoidable byproducts.
(2) Benzyl and dimethylmaleoyl (DMM) as permanent protecting groups worked reliably.
(3) Fmoc and phenoxyacetyl (PA) as temporary protecting groups permitted convenient chain extension and branching.
(4) The benzyl benzoate spacer-linker was particularly suitable; it was stable towards all reaction conditions and it permitted convenient product cleavage from the resin.
(5) Products were quite pure, for purification only standard flash chromatography or medium pressure liquid chromatography (MPLC) on silica gel were required.
(6) Overall yields even in the synthesis of complex-type *N*-glycans were very high (89 to 97% per step).
(7) A combinatorial approach to the synthesis of oligosaccharide libraries is available.
(8) The versatility of this method could be demonstrated by its successful application to the synthesis of various types of oligosaccharides.
(9) Automated SPOS was successful.

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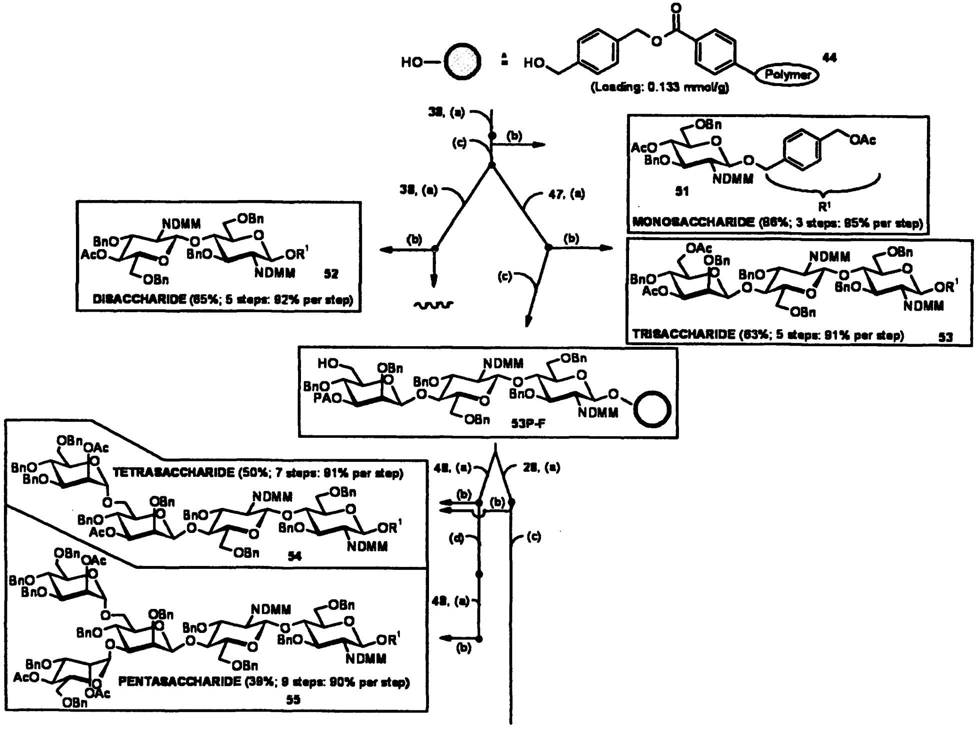
HO
HO
O
O
Polymer
44
(Loading: 0.133 mmol/g)
38, (a)
(c)
(b)
38, (a)
47, (a)
(b)
(b)
(c)
AcO
BnO
OBn
O
O
NDMM
OAc
51
R¹
MONOSACCHARIDE (86%; 3 steps: 95% per step)
BnO
AcO
NDMM
OBn
O
BnO
OR¹
NDMM
OBn
52
DISACCHARIDE (65%; 5 steps: 92% per step)
OAc
OBn
BnO
AcO
O
BnO
NDMM
O
OBn
BnO
OR¹
NDMM
OBn
TRISACCHARIDE (63%; 5 steps: 91% per step)
53
HO
BnO
PAO
OBn
O
BnO
NDMM
OBn
BnO
O
NDMM
OBn
53P-F
48, (a)
28, (a)
(b)
(b)
(d)
(c)
48, (a)
(b)
OBn
OAc
BnO
BnO
O
TETRASACCHARIDE (50%; 7 steps: 91% per step)
OBn
BnO
AcO
BnO
NDMM
OBn
BnO
OR¹
NDMM
OBn
54
OBn
OAc
BnO
BnO
OBn
BnO
BnO
NDMM
OBn
BnO
OR¹
NDMM
OBn
BnO
AcO
OAc
OBn
PENTASACCHARIDE (38%; 9 steps: 90% per step)
55

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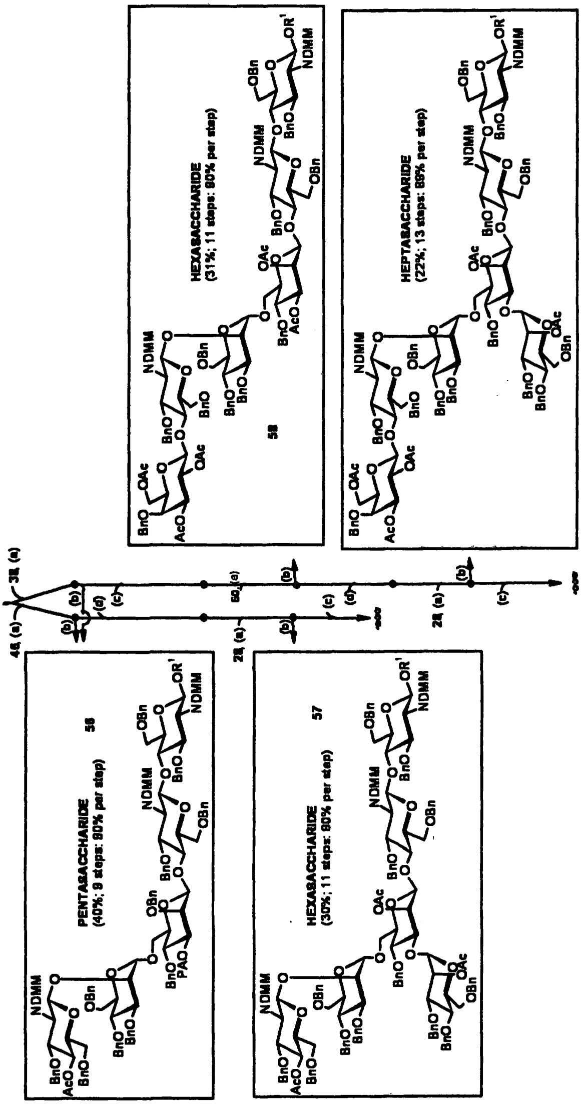
PENTASACCHARIDE
(40%; 9 steps: 90% per step)
56
HEXASACCHARIDE
(30%; 11 steps: 90% per step)
57
HEXASACCHARIDE
(31%; 11 steps: 90% per step)
58
HEPTASACCHARIDE
(22%; 13 steps: 89% per step)
NDMM
OR'
BnO
OBn
OAc
AcO
PAO
(a)
(b)
(c)
(d)
(e)

Scheme 13. SPOS of complex-type N-glycans based on a benzoate linker.

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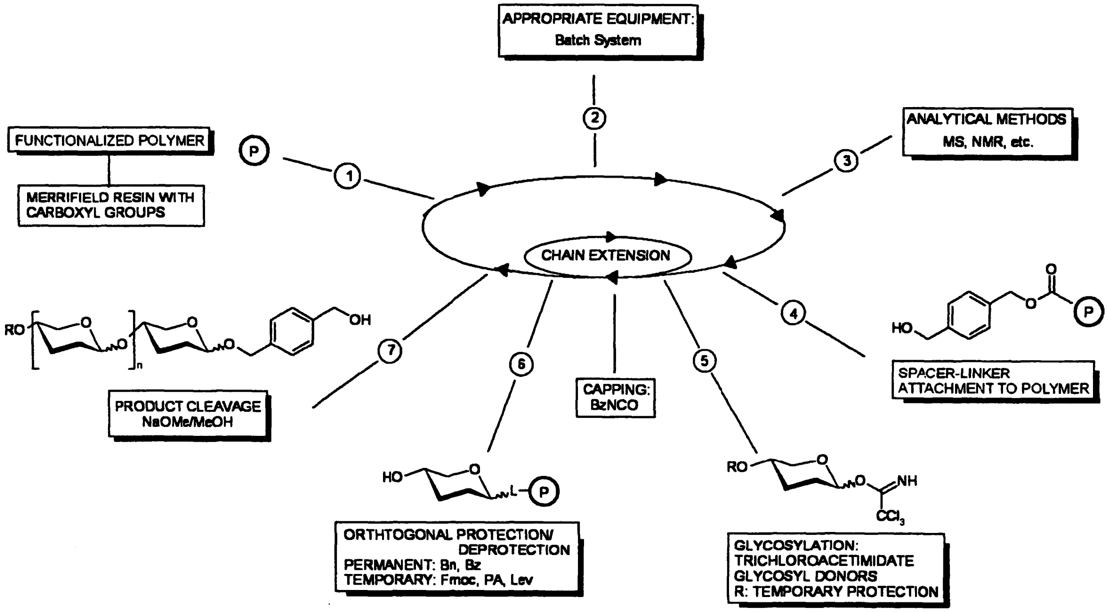
APPROPRIATE EQUIPMENT:
Batch System
FUNCTIONALIZED POLYMER
MERRIFIELD RESIN WITH CARBOXYL GROUPS
P
①
②
③
ANALYTICAL METHODS
MS, NMR, etc.
CHAIN EXTENSION
RO
O
O
O
n
O
OH
⑦
⑥
CAPPING:
BzNCO
⑤
④
HO
O
O
P
SPACER-LINKER ATTACHMENT TO POLYMER
PRODUCT CLEAVAGE
NaOMe/MeOH
HO
O
L
P
ORTHTOGONAL PROTECTION/ DEPROTECTION
PERMANENT: Bn, Bz
TEMPORARY: Fmoc, PA, Lev
RO
O
O
NH
CCl3
GLYCOSYLATION:
TRICHLOROACETIMIDATE GLYCOSYL DONORS
R: TEMPORARY PROTECTION

Scheme 14. Schematic representation of the benzoate linker SPOS.

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Chapter 14

Synthesis of Carbohydrate Antigens Related to *Shigella dysenteriae* Type 1 and of Their Protein Conjugates

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Shigella dysenteriae type 1 is a major cause of dysentery worldwide but there is no licensed vaccine against these bacteria. The lipopolysaccharide of *S. dysenteriae* type 1 is both an essssential virulence factor and a protective antigen. We describe synthesis of oligosaccharides corresponding to the repeating subunits of the O-specific polysaccharide followed by an efficient method for their covalent binding to the protein carrier, in order to obtain a well defined synthetic glycoconjugate vaccine.

In Frontiers in Modern Carbohydrate Chemistry; Demchenko, A.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2007.

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Shigellae are Gram-negative bacteria, pathogens to humans only, that can cause endemic and epidemic dysentery worldwide, especially in the developing countries (*1*). The symptoms usually start with watery diarrhea that later develops into dysentery, characterized by high fever, blood and mucus in the stool, and cramps. Dysentery may also contribute to stunted growth. Amongst Shigellae, the highest mortality rate results from *S. dysenteriae* type 1, which can cause fatalities in all age groups (*2*). Control of the spread of this disease is hampered by the low infectious dose (~ 100 bacteria). Because of increasing resistance of Shigellae to available antibiotics, development of other approaches to control this pathogen is of high priority. Attempts to use inactivated whole cell Shigella vaccines failed to result in long-term protection, and despite its discovery a century ago, there is still no licensed vaccine against *S. dysenteriae* type 1 (*1*). The major surface antigens on virulent *Shigella* are the lipopolysaccharides (LPS's). Their outer domains, termed O-specific polysaccharides (O-SP), shield the bacteria from serum complement, thus O-SP's are protective antigens, and virulence factors. The levels of antibodies to the LPS of *Shigella* spp were shown to correlate with protection against infection (*1*). Robbins and co-workers hypothesized that serum antibodies to the O-SP of Shigellae may provide lasting, protective immunity against homologous bacteria in humans (*3*). To test this hypothesis, experimental vaccines were formulated consisting of protein conjugates of the O-SP of *S. dysenteriae* type 1, *S. sonnei,* and *S.* flexneri 2a (*3*, *4*). These conjugates elicited O-SP-specific antibodies in humans. Due to our ignorance of structure-activity relationships for glycoconjugate vaccines, improvement of native polysaccharide-based vaccines poses major challenges. We surmised that oligosaccharides shorter than the native polysaccharide might also be used to elicit O-SP-specific antibodies when covalently linked to an immunogenic protein (*5*). In the last decade, synthetic chemistry has developed to a level that permits the construction of extended oligosaccharides in sufficient quantities, and in recent years, our attention has focused on the synthesis of extended oligosaccharides corresponding to the O-SP of *S. dysenteriae* type 1 and their covalent attachment to proteins to convert the non-immunogenic oligosaccharides to immunogenic glycoconjugates. Here, we describe our approach to the targeted synthetic glycoproteins (*5*, *6*, *7*).

The O-SP of *S. dysenteriae* type 1 consists of approximately 25 copies of the tetrasaccharide repeating unit **1**, which contains α-linked L-rhamnose, D-galactose, and D-*N*-acetyl-glucosamine moieties (*8*).

$$[3)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow2)\text{-}\alpha\text{-D-Gal}p\text{-}(1\rightarrow3)\text{-}\alpha\text{-D-Glc}p\text{NAc-}(1\rightarrow3)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow]_n$$

1

Retrosynthetic analysis indicated that extended fragments of the O-SP can best be constructed by block synthesis using tetrasaccharide units. Of the four possible frame-shifted sequences, we have selected the frame shown by **1**. The reason for this was that formation of an α-rhamnosyl linkage usually proceeds with higher stereoselectivity than either α-galactosylation, or α-*N*-acetyl-glucosaminylation. Moreover, the reactivity of the HO-3 hydroxyl group of rhamnose is usually higher than that of the other linkage positions.

We designed tetrasaccharide **21** as one of the building blocks for the higher-membered oligosaccharides. This unit features five lipid-type protecting groups for use as lipophilic tags that make possible the purification of such intermediates by reverse-phase, silica gel chromatography (*6*, *9*). Compound **21** contains a spacer moiety for covalent attachment of the unprotected oligosaccharide products to proteins and has a free hydroxyl group for chain extension. Tetrasacharide **21** was assembled from four monosaccharide blocks. As the first step of the oligosaccharide assembly, rhamnose acceptor **2** was reacted with the azido-glucosyl bromide **3** in the presence of silver trifluoromethanesulfonate and 2,6-di-*t*-butyl-4-methylpyridine, to afford the required, α-linked disaccharide **4** in 57 % yield (Scheme 1).

Attempted use of other donors, including the corresponding phenylthio azido-glucose or azido-glucosyl chloride failed to improve the yield or stereoselectivity of this condensation. Therefore, we have tried several other donors having various protecting group combinations, including protection of the HO-4 and HO-6 groups by isopropylidene and benzylidene acetals. These experiments failed to increase the yield of the required, α-linked disaccharide. It appeared that benzophenone ketal **6** prepared from the triol **5** by reaction with benzophenone dimethyl acetal followed by chloroacetyl anhydride, might improve this situation. Indeed, we found much improvement when the benzophenone ketal **6** was used as the donor in the presence of the activators N-iodosuccinimide and trimethylsilyl trifluoromethanesulfonate (Scheme 2). Disaccharide **7** was isolated in 90 % yield and the proportion of the undesired, β-linked disaccharide was less than 5 %. Similarly, reaction of the donor **6** with methyl rhamnoside **8** afforded the α-linked disaccharide in high yield, with a good α/β product ratio. In comparison, when the corresponding benzylidene acetal **9** was used as the donor, the yield of the α-linked disaccharide was somewhat less with a slightly higher proportion of the unwanted, β-linked disaccharide when employing the same activators (Table I).

We have also tested the stereodirecting effect of the benzophenone moiety in α-glucosylation, using thioglucosides as donors that were activated with N-iodosuccinimide and trimethylsilyl trifluoromethanesulfonate. In Table II, the data show excellent yields of the desired disaccharides, combined with high α-stereoselectivity. Interestingly, when the donors **10** and **12** were used in a less than equimolar amount, the α-selectivity increased (items 1, 3) relative to the experiments having the donor **10** and acceptors **11** and **14** in a 3:1 molar ratio (items 2, 5). The benzophenone ketal-protected donors **10** and **12** (items 1, 3)

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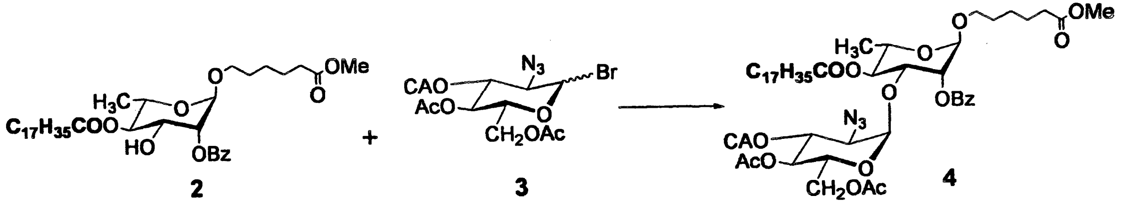
C17H35COO
H3C
O
HO
OBz
O
OMe
O
2
+
CAO
AcO
N3
Br
O
CH2OAc
3
C17H35COO
H3C
O
O
OBz
OMe
O
N3
CAO
AcO
O
CH2OAc
4

Scheme 1

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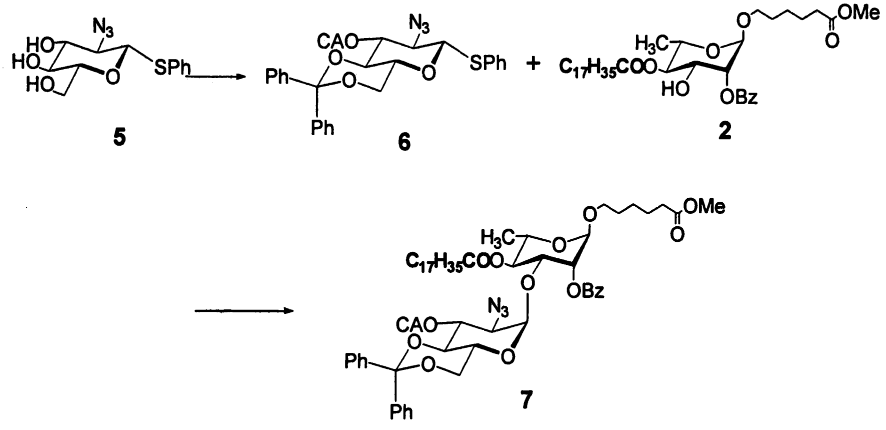

Scheme 2

consistently gave improved yields and higher α-stereoselectivity than the corresponding benzylidene acetals **13** and **16** (items 4, 8). The importance of the nature of the leaving group in influencing the stereoselectivity of glycosylation is clearly shown by the observation that reaction of the acceptor **14** with the chloride **15** resulted in poor α/β selectivity (item 6).

Replacement of the ketal protection in the disaccharide 7 by acetyls was carried out in a two step procedure. The ketal protection was removed by treatment with a mixture of acetic acid and hydrochloric acid, then the free hydroxyl groups were acetylated (Ac_2O/pyridine) to afford the disaccharide 4 in 95 % yield, from which the disaccharide acceptor 17 was obtained in a nearly quantitative yield by treatment with thiourea (Scheme 3). Further chain elongation with the lipid-protected galactosyl donor 18 afforded the desired trisaccharide in a high yield and exclusive, α-stereoselectivity. It is important to note that a similar reaction between the galactosyl donor 18 and a congener of the disaccharide 17 in which the azido function is replaced by an acetamido moiety, gave a lower yield with a decreased proportion of α-interglycosidic linkage. Therefore, the azido → acetamido conversion was carried out after the galactosylation step.

Next, the benzyl group was removed by catalytic hydrogenolysis to afford the lipidated trisaccharide acceptor **19**. The synthesis of the tetrasaccharide repeating unit **21** was completed by reaction of **19** with lipidated rhamnosyl donor **20** (Scheme 3).

The synthesis of the tetrasaccharide donor 22 is based on connections described above, except that the monosaccharide units feature conventional protecting groups. Thus, the four building blocks Shown in Scheme 4 were combined in a stepwise manner to afford the tetrasaccharide intermediate, from

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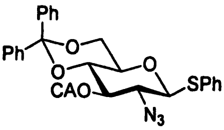
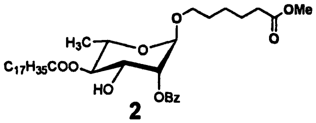
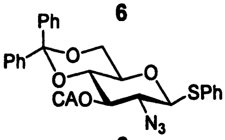
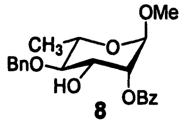
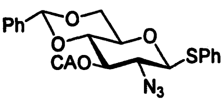
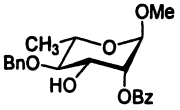
Table I. 4,6-*O*-Benzophenone ketals as stereodirecting moieties in 1,2-cis glucosaminylation

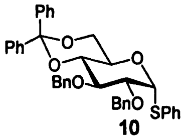
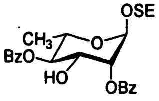
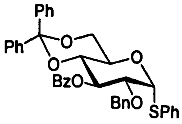
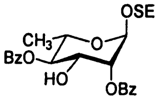
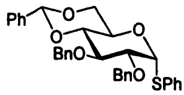
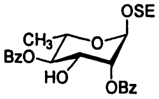
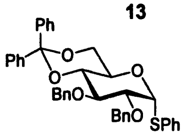
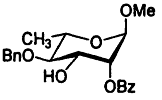
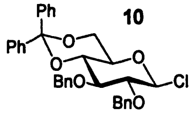
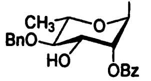
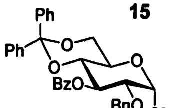
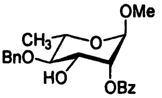
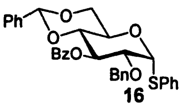
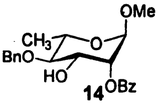
Donor	*Acceptor*	*Donor/acceptor ratio*	*α/β Product ratio*
 **6**	 **2**	3:1	95:5
 **6**	 **8**	3:1	92:8
 **9**	 **8**	3:1	87:13

which the silyl aglycon was removed by acid hydrolysis, followed by reaction with trichloroacetonitrile to afford the imidate 22. Next, a convergent approach to higher-membered oligosaccharides was undertaken. First, the tetrasaccharide acceptor **21** and the tetrasaccharide donor **22** were condensed under promotion by trimethylsilyl trifluoromethanesulfonate. The reaction mixture was applied to a C-18 reverse phase silica gel column, from which sequential elution with methanol, ethanol, and 2-propanol afforded the octasaccharide **23** in pure form, in 84 % yield, without the need for silica gel chromatography. Selective removal of the chloroacetyl group at the site of the chain extension in **23**, followed by two more cycles of glycosylation with the tetrasaccharide donor **22**, used in fourfold molar excess, afforded protected dodeca- and hexadeca-saccharides (Scheme 5). Removal of the protecting groups from the spacer-linked tetra-, octa-, dodeca-, and hexadeca-saccharides by successive exposure to NaOMe in MeOH/CH_2Cl_2 then to hydrogenolytic conditions (H_2/Pd-C) afforded the unprotected haptens **24, 25, 26,** and **27.**

Using the building blocks described above, a panel of oligosaccharides ranging from hexa- to trideca-saccharides (**28, 29, 30, 31, 32, 33**) have also been prepared as their 5-methoxycarbonylpentyl glycosides.

The oligosaccharides synthesized in this project will be used to map their immunogenicity as a function of chain length and the identity of the non-reducing terminal residue. These non-immunogenic oligosaccharide haptens can be converted to immunogenic species by covalent attachment to immunogenic

Table II. 4,6-*O*-Benzophenone ketals as stereodirecting moieties in 1,2-cis glucosylation

Item	*Donor*	*Acceptor*	*Donor/acceptor ratio*	*α/β Product ratio*
1	 **10**	 **11**	0.5	α only
2	**10**	**11**	3:1	95:5
3	 **12**	 **11**	0.5	α only
4	 **13**	 **11**	0.5	95:5
5	 **10**	 **14**	3:1	92:8
6	 **15**	 **14**	2:1	4:1
7	 **12**	 **14**	3:1	95:5
8	 **16**	 **14**	3:1	88:12

proteins (*10*). In order to avoid denaturation of the protein, the conjugation has to be performed in an aqueous solution, near physiological pH, at ambient temperature, preferably in a short time (*11*, *12*). Because of the expensive nature of oligosaccharide synthesis, the conjugation should be high-yielding, and the recovery of unreacted hapten in its original form is also desirable. We have recently proposed a new, efficient, and mild protocol for the covalent coupling of saccharides to proteins (*13*). The approach is based on oxime formation between an *O*-alkylhydroxylamine and aldehydo or keto groups.

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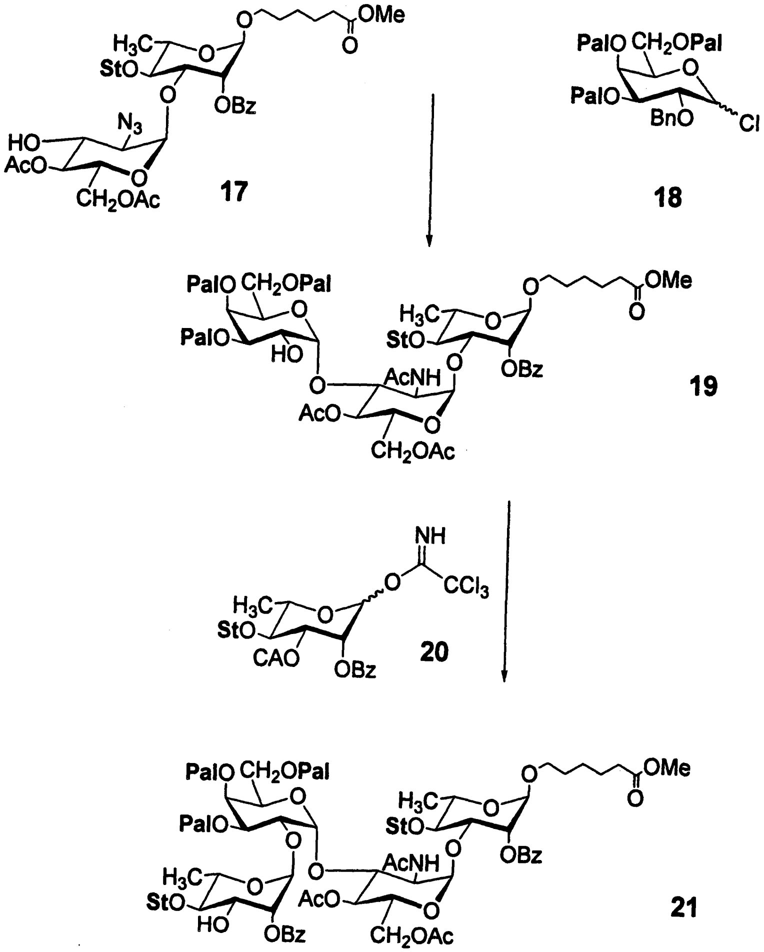
OMe
O
H$_3$C
O
StO
O
OBz
N$_3$
HO
AcO
O
CH$_2$OAc
17
PalO CH$_2$OPal
O
PalO
BnO
Cl
18
PalO CH$_2$OPal
O
PalO
HO
O
AcNH
AcO
CH$_2$OAc
H$_3$C
StO
OBz
OMe
19
NH
O
CCl$_3$
H$_3$C
StO
CAO
OBz
20
PalO CH$_2$OPal
PalO
H$_3$C
StO
HO
OBz
AcNH
AcO
CH$_2$OAc
OMe
21

Pal = palmitoyl **St** = stearoyl

Scheme 3

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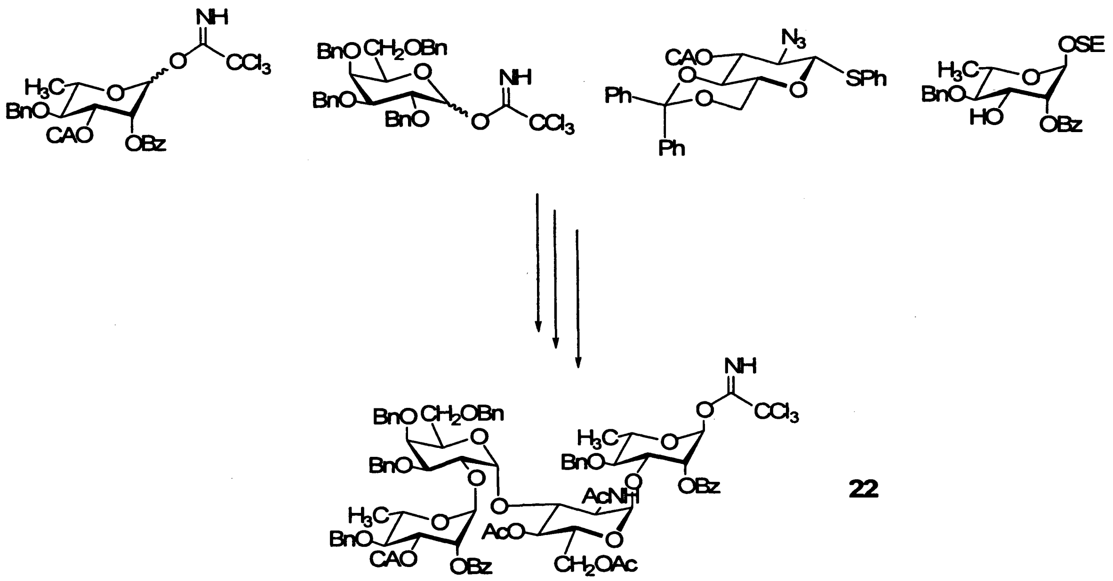
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CCl3
H3C
BnO
CAO
OBz
BnO CH2OBn
BnO
BnO
NH
CCl3
CAO
N3
Ph
Ph
SPh
H3C
OSE
BnO
HO
OBz
NH
CCl3
BnO CH2OBn
BnO
H3C
BnO
OBz
AcNH
H3C
BnO
CAO
OBz
AcO
CH2OAc

22

Scheme 4

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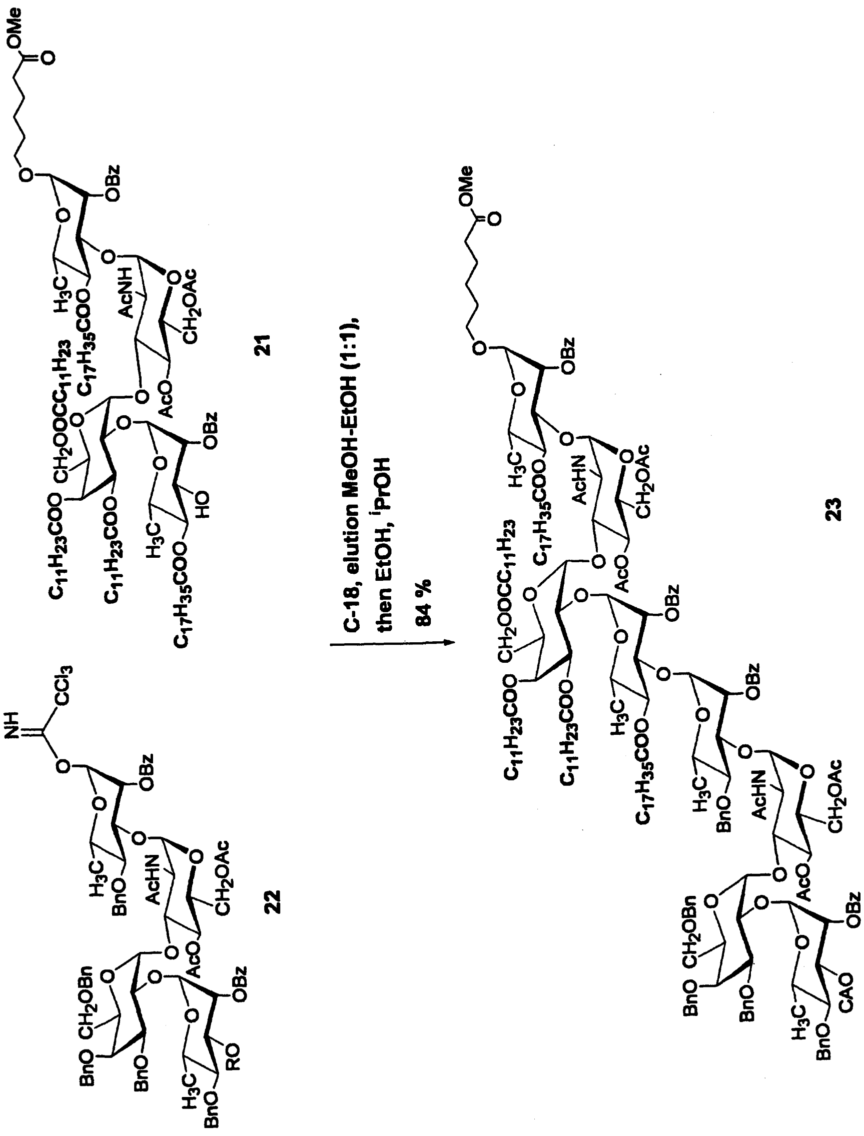
21
22
23
C-18, elution MeOH-EtOH (1:1),
then EtOH, ^{i}PrOH
84 %
OMe
O
OBz
AcNH
AcHN
CH$_2$OAc
AcO
HO
RO
CAO
BnO
CH$_2$OBn
H$_3$C
C$_{11}$H$_{23}$COO
C$_{17}$H$_{35}$COO
CH$_2$OOCC$_{11}$H$_{23}$
NH
CCl$_3$

Scheme 5

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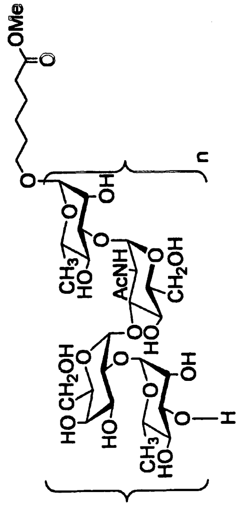
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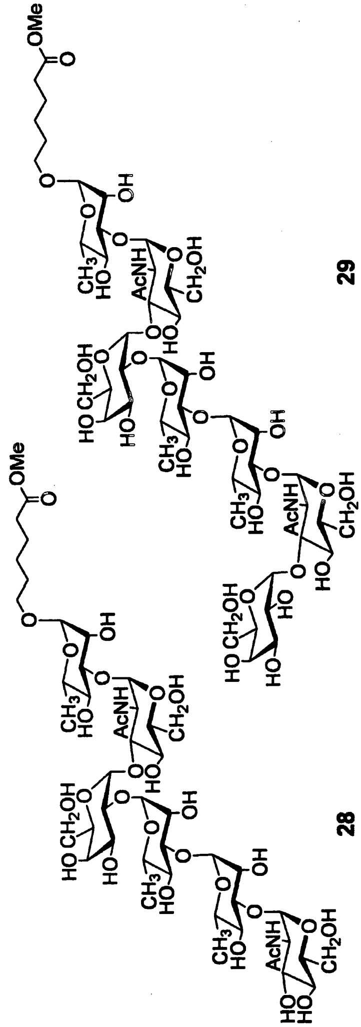
O
O
n
OH
O
CH3
HO
O
AcNH
O
CH2OH
HO CH2OH
O
HO
O
HO
OH
O
CH3
HO
O—H

24 n = 1
25 n = 2
26 n = 3
27 n = 4


OMe
O
O
OH
CH3
HO
AcNH
CH2OH
HO CH2OH
HO
OH
CH3
HO
HO
HO

28

29

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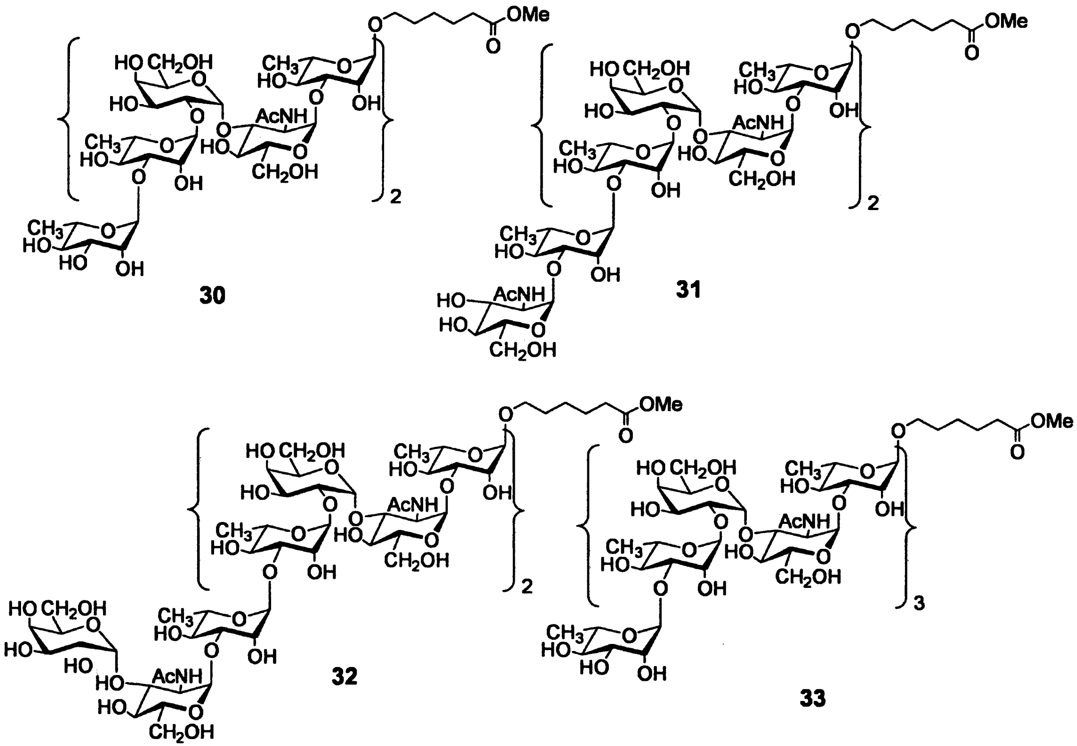
HO CH$_2$OH
HO
O
CH$_3$
HO
OH
AcNH
HO
CH$_2$OH
OMe
2
3

30

31

32

33

Chemical structures of synthesized compounds

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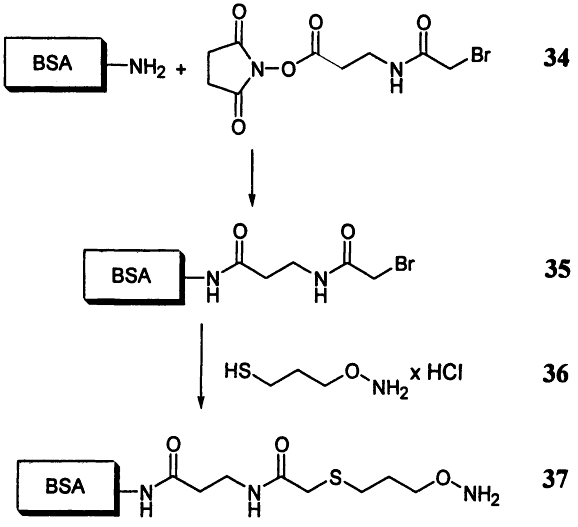

Scheme 6

According to our protocol, the protein is derivatized with a spacer carrying an aminooxy group, whereas the carbohydrate part is equipped with an aldehydo or a keto group in its aglycon. Aminooxy groups have been attached to bovine serum albumin (BSA) in a two-step procedure, as shown in Scheme 6. First, reactive bromoacetyl groups were introduced in the protein, using the commercially available reagent **34**. Next, the derivatized protein **35** is treated with the heterobifunctional linker **36** to afford the aminooxylated BSA **37**. Under our conditions, an average of 30-35 aminooxy moieties were introduced to BSA, as estimated by molecular weight determinations using MALDI-TOF mass spectrometry with sinapinic acid as the matrix. Keto groups were introduced in the spacer-linked saccharides, as shown in Scheme 7. The ester **24** was treated with 1,2-diaminoethane to afford the corresponding amide **38**. Next, the amide was acylated with 5-oxohexanoic anhydride **39** to yield the keto derivative **40** in a quantitative yield. Coupling of the aminooxy-modified BSA **37** with the keto hapten **40** took place in a pH 6.5 buffered solution by incubation for 12 h at 22 °C. Small molecules were removed by gel filtration through a Sephadex G-50 column, from which the void volume fractions were collected and analyzed by MALDI-TOF mass spectrometry.

Starting from BSA having an average of 34 aminooxy groups, and using tetrasaccharide **40** in an approximately equimolar amount relative to the aminooxy groups available on the protein, the average number of incorporated haptens was 17. Any unbound ligand could be recovered unchanged.

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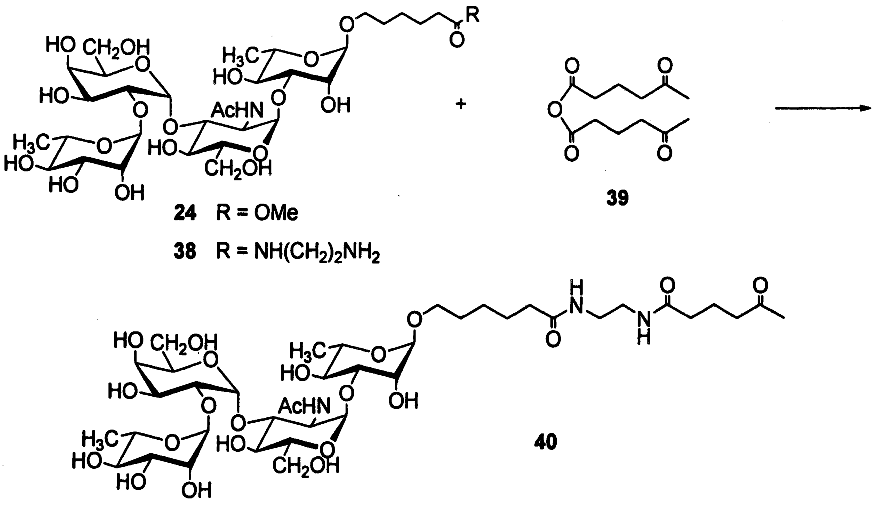

Scheme 7

Conclusions

We have developed a convergent synthetic strategy to prepare oligosaccharides of increasing complexity, representing up to four consecutive tetrasaccharide repeating units of the O-specific polysaccharide of *Shigella dysenteriae* type 1. We have taken advantage of lipid protecting group-functionalized glycosyl acceptors that enabled efficient purification of the protected intermediates using reverse-phase chromatography. We have designed an oxime chemistry-based conjugation protocol for bioconjugation and demonstrated its utility for linking both neutral and charged saccharides to proteins. In our new protocol unreacted haptens can be recovered unchanged and may be reused. These saccharide-protein constructs are currently being used in immunization experiments aimed at estimating the role of structural factors in oligosaccharide immunogenicity.

Acknowledgment

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Chapter 15

Enzymatic Synthesis of Glycosaminoglycans: Improving on Nature

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Chapel Hill, NC 27599

The chemoenzymatic synthesis of glycosaminoglycan (GAG) analogs is described. This chapter is divided into three main sections, each describing the use of one enzyme family in GAG synthesis. First, the polysaccharide lyase enzymes are described. These enzymes play crucial role in obtaining GAG oligosaccharides. Second, GAG synthases, bacterial enzymes that transfer UDP-monosaccharides to acceptor oligosaccharide are described, to obtain homogenous oligosaccahrides and polysaccharides. Finally, GAG sulfotransferases, that play a major role in the biosynthesis, are described for the introduction of sulfo groups to the appropriate positions on the polysaccharide backbone.

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1. INTRODUCTION

Glycosaminoglycans (GAGs) are highly charged polysaccharides consisting of repeating 1→3 and/or 1→4 linked hexosamine and uronic acid residues (**Figure 1**).[1, 2] GAGs are polydisperse mixtures of polysaccharide chains of varying lengths with an average molecular weight of 10^4-10^6 Da.[2] GAGs are synthesized by both prokaryotes[3] and eukaryotes and are found in all animal cells.[2] Pharmaceutical heparin is an example of a structurally complex GAG that is prepared from porcine intestinal mucosa and bovine lung in amounts of 30 metric tons/yr. worldwide.[4]

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1.1. GAG-Protein Binding, Biological Activities and Therapeutic Significance.

GAGs are highly charged polyanions that interact with hundreds of different proteins.[1, 5-7] GAGs bind to proteins primarily through the interaction of their sulfo and carboxyl groups with basic amino acid residues (*i.e.*, arginine and lysine) present in shallow pockets or on the surface of GAG-binding proteins.[1, 5, 6] For example, specific sequences in the heparin molecule, bind to antithrombin (AT) inhibiting a number of physiologically important serine proteases.[8-10] In the past twenty years GAGs have been shown to play a central role in the regulation of a large number of important cellular processes including cell growth and cell-cell interaction.[6, 11, 12] The ability of GAGs, such as heparin and heparan sulfate, to regulate mitogenesis and cell migration primarily results from their interaction with growth factors and chemokines.[7, 13, 14] The exploitation of specific GAG-protein interactions might lead to important new therapeutic advances. For example, specific oligosaccharide sequences within GAGs, interacting with growth promoting proteins (*i.e.*, fibroblast growth factors (FGF),[14-18] vascular endothelial growth factor (VEGF),[19] heparin binding growth adhesion molecule (HBGAM),[20] hepatocyte growth factor (HGF),[21] insulin-like growth factor (IGF),[22] bone morphogenic protein (BMP),[23] *etc.*), might be applied to wound healing/tissue growth[24] or to inhibiting angiogenesis in the eradication of tumors.[12, 25] GAG-binding proteins (*i.e.*, annexin,[26] antithrombin (AT),[27] heparin cofactor II,[28] coagulation factors,[29] etc.) are important in the regulation of the biochemical cascades involved in coagulation[29] and complement activation.[30] GAG-binding proteins are frequently found on the surface of pathogens (*i.e.*, malaria circumsporozoite protein,[31, 32] hepatitis C virus,[33] herpes simplex virus glycoproteins,[34] human immunodeficiency virus (HIV)[35] and dengue envelope

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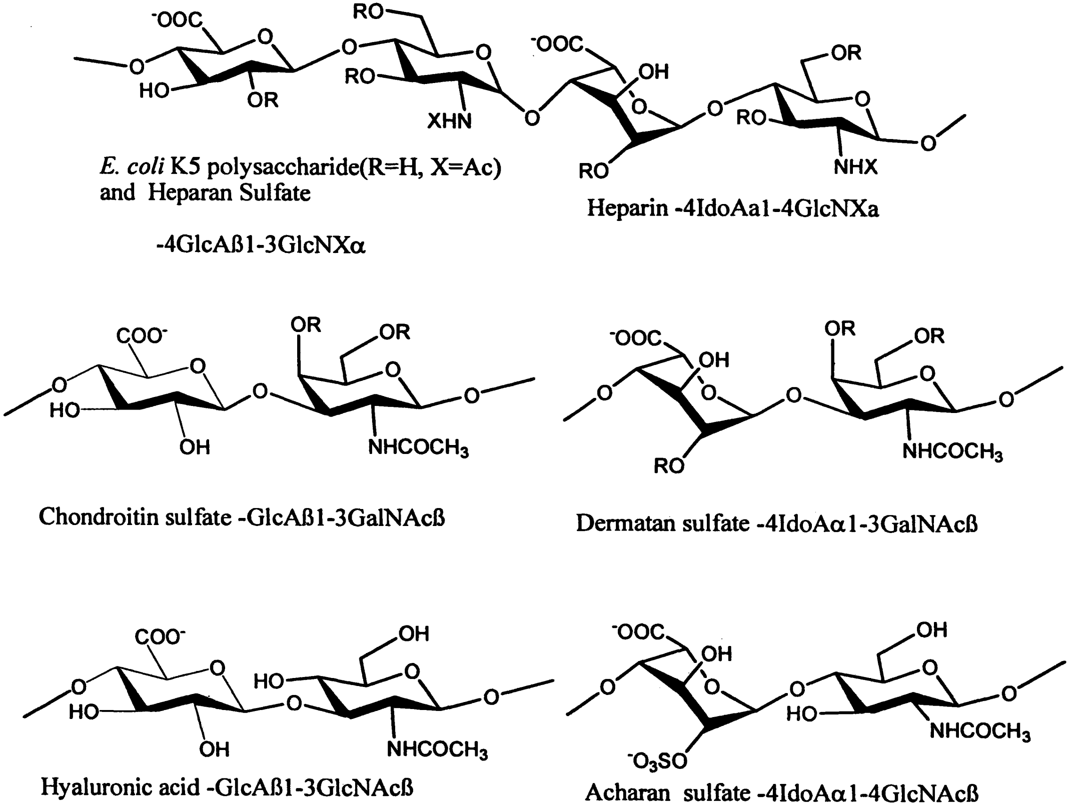
-OOC
RO
O
HO
OR
RO
XHN
O
-OOC
OH
O
RO
OR
NHX
COO-
OR
OR
HO
OH
NHCOCH3
-OOC
OH
RO
OR
OR
NHCOCH3
COO-
OH
HO
HO
OH
NHCOCH3
-OOC
OH
-O3SO
OH
HO
NHCOCH3

E. coli K5 polysaccharide(R=H, X=Ac) and Heparan Sulfate

-4GlcAß1-3GlcNXα

Heparin -4IdoAa1-4GlcNXa

Chondroitin sulfate -GlcAß1-3GalNAcß

Dermatan sulfate -4IdoAα1-3GalNAcß

Hyaluronic acid -GlcAß1-3GlcNAcß

Acharan sulfate -4IdoAα1-4GlcNAcß

Figure 1. GAG structure (R = H or SO_3^-, X = COCH3, or SO_3^- or H)

protein[36]) facilitating their attachment to and infection of mammalian cells.[7] The structure, physiochemical characterization, and many biological activities of GAGs have been reviewed in detail.[1, 2, 4-6, 8, 9, 11, 37-39] While all GAGs show important biological activities, this chapter focuses primarily on heparin and heparan sulfate glycosaminoglycans.

Heparin binds to AT, a serine protease inhibitor, which becomes activated and inhibits thrombin and other biologically important serine proteases.[9] Rosenberg *et al.*[40] and Lindahl *et al.*[41] deduced the structure of the AT-binding site (**Figure 2**), by performing a partial depolymerization of heparin, and then purified the products using affinity chromatography on immobilized antithrombin.

The presence of an unusual 3-*O*-sulfo group on a glucosamine residue was demonstrated by the release of sulfate on the incubation of a pentasaccharide with 3-*O*-sulfatase[42] and confirmed by NMR studies.[43] Chemical synthesis of a pentasaccharide containing this unique 3,6-di-*O*-sulfoglucosamine residue substantiated these findings.[44] This chemical synthesis also facilitated detailed structure-activity relationship (SAR) studies on heparin, where numerous analogs were synthesized and their binding properties tested.[45] Such time consuming, and intensive studies, while scientifically valuable, are simply not possible for oligosaccharides corresponding to each of the over 100 proteins that bind heparin.[5-7]

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1.2. Synthesis of Defined GAG Oligosaccharides

Despite recent advances,[46] the total chemical synthesis of GAGs, GAG oligosaccharides and derivatives using current state of the art techniques, has serious limitations due to the heteregeneity of GAG structures, especially in sulfation pattern (**Table 1**) and the configuration of uronic acids.[38, 46] The multiple steps required to chemically synthesize an intricately substituted carbohydrate, while displaying elegant chemistry,[45-47] results in a product that simply costs too much. Indeed, much synthetic work has focused on simplifying the target structure (*i.e.*, replacing 2-amino-2-deoxy-D-glucopyranose with D-glucopyranose to prepare active analogs) rather than optimizing the synthesis of the natural product.[45, 48] While recent advances in the chemical synthesis of GAG oligosaccharides by Jacquinet,[49, 50] Jan Boons,[51] Seeberger,[52] and others[53, 54] have decreased the number of synthetic steps, increased stereocontrol and enhanced yields, each new target still represents a major research commitment. Despite problems inherent to the chemical synthesis of GAG oligosaccharides, the synthetic AT-binding pentassacharide Arixtra, has been successfully introduced as a clinical anticoagulant in both the U.S. and Europe.[55] There are several reasons driving the use of this pentasaccharide including: 1) The

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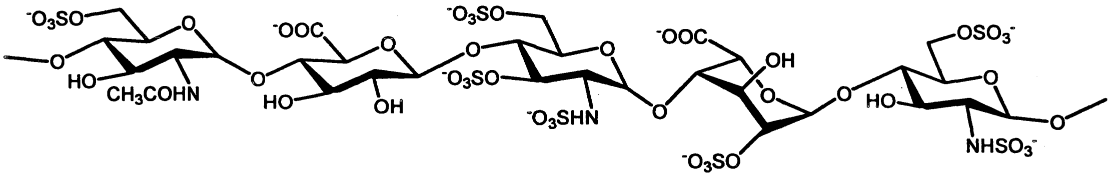
^-O_3SO
^-OOC
^-O_3SO
^-OOC
OH
OSO_3^-
HO
CH_3COHN
HO
OH
^-O_3SO
^-O_3SHN
^-O_3SO
HO
$NHSO_3^-$

Figure 2. Structure of the AT binding site of heparin/ heparan sulfate

perceived benefits of a pure, homogenous anticoagulant/antithrombotic agent;[6, 38] 2) The clinical failure of other synthetic non-heparin anticoagulant/ antithrombotic agents;[56] and 3) The concern, particularly in Europe, about difficulties in establishing that heparin is prion-free.[57]

Table 1. Most encountered sulfation patterns in GAGs

Heparin/Heparan sulfate	Chondroitin sulfate	Dermatan sulfate
GlcN6S	GalN6S	GalN4S
GlcNS6S	GalN4S	
GlcNS3S6S	GalN4,6S	
IdoA2S	GlcA2S	IdoA2S
GlcA3S	GlcA3S	

In the past decade all the heparin biosynthetic enzymes have been isolated, cloned and expressed.[58] These enzymes, while still incompletely characterized, have recently been used to prepare the AT-binding site.[59, 60] This technical achievement resulted in only ~1 μg of AT-binding site and is also limited to the preparation of only naturally occurring structures. A new chemoenzymatic synthetic approach, such as that being developed in our laboratories, affording multimilligram amounts of GAG oligosaccharides with a variety of natural and unnatural structures is needed.

This chapter describes recent advances in chemoenzymatic preparation of GAG analogs. Three enzyme families, GAG lyases, synthases and sulfotransferases, are described for the preparation of natural and unnatural GAG analogs.

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2. POLYSACCHARIDE LYASES

The Linhardt laboratory prepares polysaccharide lyases from *Flavobacterium heparinum*[61] and *Bacteroides stearcoris.*[62] These enzymes have been purified to homogeneity, cloned, and expressed in bacteria. Their physical and catalytic properties and specificity have been extensively investigated.[61, 63-67]

2.1 Mechanism of lyase catalyzed GAG depolymerization

Microorganisms utilize an eliminative mechanism to breakdown GAGs that involves abstraction of the proton at C-5 of the hexuronic acid by a general base and β-elimination of the 4-O-glycosidic bond with concomitant formation of an unsaturated C4-C5 bond within the hexuronic acid located at the non-reducing end (**Figure 3**).[68] The leaving group must be protonated, either by a side chain acting as a general acid, or by proton abstraction from a water molecule. Proton abstraction and β-elimination are expected to proceed in a stepwise as opposed to concerted manner.[69, 70]

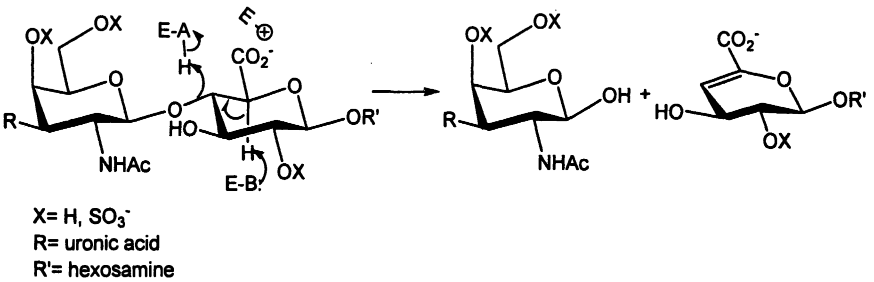

Figure 3. Eliminative cleavage mechanism for chondroitin lyase family. *R = GlcAp; R' = GalpNAc; X = H or SO_3^- (where E = enzyme; A = an acidic group on the enzyme capable of protonating the glycosidic oxygen; B = a basic residue capable of removing the acidic proton from the C-5 of the GlcAp residue; and "+" a positive amino acid residue or a metal ion capable of favorably interacting with (stabilizing) the negative charge formed at the carboxyl group.*

There is an extensive variation in specificity among lyases for different GAG types. Thus, chondroitinase B is specific for cleavage of dermatan sulfate, accepting only an iduronic acid, whereas chondroitinase ABC will accept either glucuronic acid or iduronic acid (**Table 2**). Extensive biochemical and mutagenesis studies have been carried out on enzymes obtained from *Flavobacterium heparinum* (*Pedobacter heparinus*), which produces three heparin lyases[61] and two chondroitinases (FlavoAC and FlavoB)[70, 71] and on two general specificity chondroitinases from *Proteus vulgaris* (PvulABCI and PvulABCII).[72]

The Linhardt laboratory also prepares chondroitin sulfate lyases from *F. heparinum*. These enzymes have been purified to homogeneity, cloned, expressed, and the physical and catalytic properties and specificity of chondroitin B lyase and chondroitin AC lyase have been extensively investigated.[64-68, 71] Chondroitin lyases act on chondroitin sulfates, dermatan sulfate, and hyaluronic acid and can afford large quantities of oligosaccharides for use as inexpensive building blocks.[73] Some of the well characterized polysaccharide lyases acting on glycosaminoglycans are listed in **Table 2**.

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Table 2. Properties of polysaccharide lyases acting on glycosaminoglycans

Name[a]	Substrates	Linkage specificity[b]	Action pattern	Mr (Da)
Heparinase I (Fh)[61, 63, 74, 75]	Heparin HS	→4)GlcNS6X(1→4)IdoA2S(1→	Endo	42,800
Heparinase II (Fh)[61, 63, 74, 75]	Heparin HS	→4)GlcNY6X(1→4)UA2X(1→	Endo	84,100
Heparinase II (Bs)[74, 76]	Heparin HS	→4)GlcNY6X(1→4)IdoA2S(1→	Endo	100,000
Heparinase III (Fh)[61, 63, 74, 75]	HS	→4)GlcNY6X(1→4)GlcA(1→	Endo	70,800
Chondroitinase AC (Fh)[71, 77]	CS-A (4S) CS-C (6S) HA	→3)GalNAc(or GlcNAc)4X,6X(1→4)GlcA(1→	Endo	74,000
Chondroitinase AC (Aa)[66]	CS-A (4S) CS-C (6S) HA	→3)GalNAc(or GlcNAc)4X,6X(1→4)GlcA(1→	Exo	79,840
Chondroitinase B (Fh)[71]	DS(CS-B)	→3)GalNAc4X,6X(1→4)IdoA2X(1→	Endo	55,200
Chondroitinase ABC (Bs)[78]	CS-A (4S) CS-C (6S) DS(CS-B)	→3)GalNAc4X,6X(1→4)UA2X(1→	Endo	116,000
Hyaluronate lyase (Pa)[79]	HA CS-A (4S) CS-C (6S)	→3)GalNAc(or GlcNAc)4X,6X(1→4)GlcA(1→		85,110

[a] Fh, *Flavobacterium heparinum*; Aa, *Arthrobacter aurescens*; Pv, *Proteus vulgaris*; Bs, *Bacteroides stercoris*; Pa, *Propionibacterium acnes*

[b] The primary sites of action are shown. X=SO_3Na , Y= SO_3Na or $COCH_3$, UA= glucuronic or iduronic acid.

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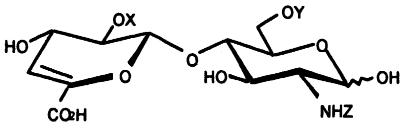
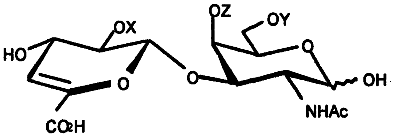
2.2 The use of polysaccharide lyases in preparation of GAG oligosaccharides

Polysaccharide lyases have been used to produce $\Delta^{4,5}$-uronate containing disaccharides (**Figure 4, 1a-1r**) and higher oligosaccharides from heparin, heparan sulfate, chondroitin sulfates, dermatan sulfate, hyaluronan, and chemically modified GAGs.[62, 68, 74, 80-82]



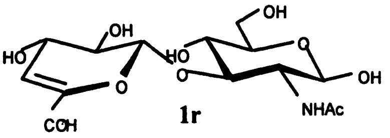
heparin/heparan sulfate disaccharides

1a $X = SO_3^-$, $Y = SO_3^-$, $Z = SO_3^-$
1b $X = SO_3^-$, $Y = H$, $Z = SO_3^-$
1c $X = H$, $Y = SO_3^-$, $Z = SO_3^-$
1d $X = H$, $Y = H$, $Z = SO_3^-$
1e $X = H$, $Y = H$, $Z = Ac$
1f $X = SO_3^-$, $Y = SO_3^-$, $Z = Ac$
1g $X = SO_3^-$, $Y = H$, $Z = Ac$
1h $X = H$, $Y = SO_3^-$, $Z = Ac$
1i $X = SO_3^-$, $Y = SO_3^-$, $Z = H_2^+$



chondroitin sulfate/dermatan sulfate disaccharides

1j $X = H$, $Y = H$, $Z = H$
1k $X = SO_3^-$, $Y = H$, $Z = H$
1l $X = H$, $Y = SO_3^-$, $Z = H$
1m $X = SO_3^-$, $Y = SO_3^-$, $Z = H$
1n $X = H$, $Y = H$, $Z = SO_3^-$
1o $X = SO_3^-$, $Y = H$, $Z = SO_3^-$
1p $X = H$, $Y = SO_3^-$, $Z = SO_3^-$
1q $X = SO_3^-$, $Y = SO_3^-$, $Z = SO_3^-$



1r

Hyaluronic acid disaccharide

Figure 4. Glycosaminoglycan disaccharides prepared in the Linhardt laboratory that will serve as synthetic building blocks. Structure 1a is the abundant heparin trisulfated disaccharide.

Because both the polysaccharide substrates and enzymes are relatively inexpensive, these oligosaccharides can be prepared in large quantities at a low cost. Indeed, the discovery of new GAGs, such as acharan sulfate[83] as well as *Escherichia coli* polysaccharide K5 (heparosan),[84] can afford further structures in large quantities and at low cost (**Figure 1**). Currently this approach, for preparing large quantities of inexpensive GAG-derived oligosaccharides, is limited by the small number of available lyases[74, 82] and their limited specificities.[63, 75, 85]

Recently we proposed that these lyase-derived oligosaccharides could be chemically linked together to form larger oligosaccharides with the requisite structure for a wide variety of biological activities.[86] The first objective would be to differentially protect enzymatically prepared desulfated disaccharides and to use these neutral disaccharides to prepare larger target oligosaccharides (**Figure 5**).[87] The advantages of this approach are 1) disaccharides can be assembled into oligosaccharides with a reduced number of glycosylation reactions and 2) a high level of structural complexity (*i.e.*, stereochemistry, sulfation pattern) is already built into these disaccharides. In addition, we investigated the use of 2,2,2-trifluorodiazoethane as a reagent for sulfo group protection in enzymatically prepared CS disaccharides (**Figure 5**).[88]

This approach was first used for sulfate ester protection in carbohydrates by Flitsch and co-workers.[89] Once the sulfo groups have been protected, the free hydroxyl and carboxyl groups could be protected in organic solvents used in standard carbohydrate synthesis. This chemistry has been successfully used to selectively protect primary and secondary *O*- and *N*- sulfo groups in unprotected sulfated mono- and di-saccharides in high yields.[88, 90]

3. GLYCOSAMINOGLYCAN SYNTHASES

Most described glycosyltransferase enzymes catalyze the transfer of only one specific type of monosaccharide to an acceptor molecule. In contrast, the various glycosyltransferases (GAG synthases) that produce the GAG polymer backbone transfer two distinct monosaccharides (HexNAc and GlcA) to the growing chain in a repetitive fashion.[3, 91] In all known organisms, the enzymes synthesize the alternating sugar repeat backbones utilizing UDP-sugar precursors and metal cofactors (*e.g.*, magnesium and/or manganese ion) near neutral pH according to the overall reaction:

$$n\text{UDP-GlcA} + n\text{UDP-HexNAc} \rightarrow 2n\text{UDP} + [\text{GlcA-HexNAc}]_n \quad \text{(Eq. 1)}$$

where HexNAc is GlcNAc (N-acetylglucosamine) or GalNAc (N-acetylgalactosamine. Depending on the specific GAG and the particular organism or tissue examined, the degree of polymerization, n, ranges from $10^{2\text{-}4}$.

The GAG synthase enzymes are found in a variety of organisms including all animals from hydras to humans, certain pathogenic bacteria, and at least one virus. However, with respect to chemoenzymatic synthesis of carbohydrates, the

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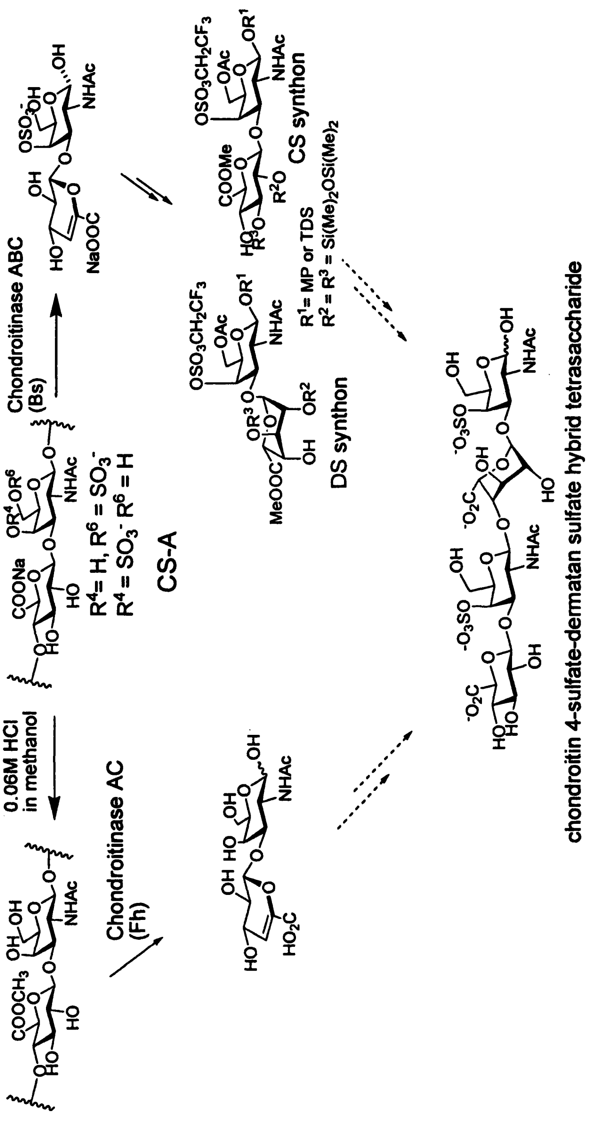
0.06M HCl in methanol
Chondroitinase ABC (Bs)
Chondroitinase AC (Fh)
CS-A
$R^4 = H, R^6 = SO_3^-$
$R^4 = SO_3^-\ R^6 = H$
DS synthon
CS synthon
$R^1 = MP$ or TDS
$R^2 = R^3 = Si(Me)_2OSi(Me)_2$
COOCH3
COONa
NaOOC
HO2C
MeOOC
COOMe
OSO3CH2CF3
NHAc
chondroitin 4-sulfate-dermatan sulfate hybrid tetrasaccharide

Figure 5. Preparation of desulfated and sulfoprotected disaccharide starting materials for the synthesis of CS/DS oligosaccharides

enzymes from the Gram-negative bacteria *Pasteurella multocida* are extremely useful because: (a) the catalysts for the production of all three uronic acid-containing GAGs are present in various serotypes of this microbe, (b) the isolated recombinant enzymes may be produced in soluble forms that either (c) rapidly form long polymer chains *in vitro* or (d) readily extend exogenously supplied GAG oligosaccharides *in vitro*.[3] In contrast, most of the other known enzymes are not as easy to use (membrane proteins obtained in poor yields), exhibit low polymerization activity after isolation, and/or cannot elongate existing polymers.

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3.1 *Pasteurella* Glycosaminoglycan Synthases

The native *Pasteurella* bacterial GAG glycosyltransferases are associated with the cell membranes; this localization makes sense with respect to synthesis of polysaccharide molecules destined for the cell surface to form the sugar coating known as a capsule. It appears that these enzymes associate with other membrane components of the Gram-negative capsular polysaccharide transport apparatus and, fortunately, are themselves not integral membrane proteins. The *Pasteurella* native sequence catalysts possess very good sugar transfer specificity (*i.e.* will use only the authentic UDP-sugar precursors) but will acommodate a variety of acceptor oligosaccharides (*i.e.* certain non-cognate GAG sugars may be elongated; discussed later). All the enzymes can be produced in a functional state by utilizing the appropriate expression plasmid in most laboratory strains of *Escherichia coli.*

The first *Pasteurella* GAG synthase to be identified was the 972-residue HA synthase from Type A strains, PmHAS (**Table 3**). This single polypeptide transfers both sugars, GlcNAc and GlcA, to form the HA disaccharide repeat.[3] UDP-Glc or UDP-GalNAc do not serve as substrates for this enzyme. The chondroitin chain is chemically identical to HA except that its HexNAc residue is GalNAc instead of GlcNAc. The 965-residue Type F enzyme, PmCS, which has ~90% identity at the gene and protein level to PmHAS, polymerizes unsulfated chondroitin chains.[3] This enzyme uses UDP-GalNAc, but not the C4 epimer UDP-GlcNAc, as a donor.

The 617-residue Type D *Pasteurella* heparosan synthase, PmHS1,[3] and the 651-residue Type A,D, and F *Pasteurella* cryptic heparosan synthase, PmHS2,[3] are not very similar at the protein level to either PmHAS or PmCS. Both of these enzymes produce unsulfated heparosan chains. The PmHS enzyme, however, resembles a fusion of the *E. coli* K5 KfiA and KfiC proteins[92]

Table 3. *Pasteurella* Glycosaminoglycans and Synthases

Polysaccharide*	Repeat Structure	P. multocida	enzyme
HA, Hyaluronan	[β1,4 GlcA- β1,3 GlcNAc]	Type A	PmHAS
Chondroitin	[β1,4 GlcA- β1,3 GalNAc]	Type F	PmCS
Heparosan	[β1,4 GlcA- α1,4 GlcNAc]	Type D Type A,D,F	PmHS1 PmHS2

* no bacterial polymer is known to be naturally sulfated.

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responsible for making heparosan in this human pathogen. Even though HA and heparosan polysaccharides have identical sugar compositions, the basic enzymology of their synthases must differ. All UDP-sugar precursors are alpha-linked. HA is an entirely beta-linked polymer, therefore, only inverting mechanisms are utilized during the sugar transfer process. In contrast, heparosan contains alternating alpha- and beta-linkages suggesting that both a retaining and an inverting mechanism are involved in synthesis. The production of the two types of anomeric glycosidic bonds probably requires distinct catalytic sites.

3.1.1 Domain Structures of *Pasteurella* GAG Synthases

Three-dimensional structures are not yet available for any GAG or polysaccharide synthase, but the PmHAS and PmCS enzymes have been shown to contain two independent glycosyltransferase sites by biochemical analysis of various mutants (**Figure 6**).[93, 94] The HexNAc-transferase or the GlcA-transferase activities of the Pasteurella enzyme can be assayed separately in vitro by supplying the appropriate acceptor oligosaccharide and only one of the UDP-sugar precursors (as in Equations 2 or 3). Two tandemly repeated sequence elements are present in PmHAS and PmCS. Each element contains a short sequence motifs containing aspartate-glycine-serine or aspartate-x-aspartate. Mutation of the aspartate residue in any one motif of PmHAS or PmCS converts the dual-action synthase into a single-action glycosyltransferase.[93, 94] The newly described PmHS1 and 2 also appear to possess two domains in our ongoing studies.

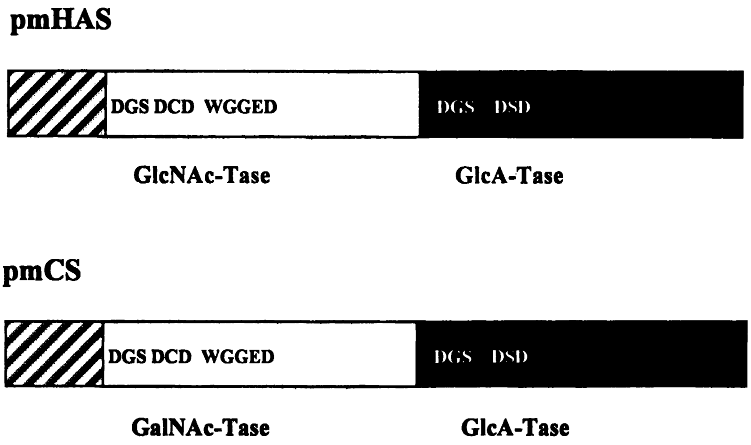

Figure 6. Schematic of PmHAS and PmCS Domains.
The truncated, soluble recombinant enzymes contain two distinct sugar transfer domains. If the aspartates (D) are mutated in one domain, then that glycosyltransferase activity is destroyed, but the other domain functions normally. The PmHS1 and 2 domain structures are still being delineated.

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3.1.2 Elongation Activity of Recombinant *Pasteurella* Synthases

The *E. coli*-derived recombinant PmHAS, PmCS, and PmHS1,2 will elongate exogenously supplied GAG-oligosaccharide acceptors *in vitro;*[3] these virgin enzymes lack a nascent GAG chain because the typical host utilized for expression lacks the required UDP-GlcA precursor. The *Pasteurella* GAG synthases add sugars to the non-reducing terminus of the linear polymer chain as determined by testing defined acceptor molecules.[3] Therefore, the reducing termini of the acceptor sugar may be modified without compromising elongation. We have found that various tagged (*e.g.*, radioactive or fluorescent) or immobilized (*e.g.*, on plastic or glass surfaces) acceptor molecules are elongated by PmHAS, PmCS, or PmHS1 (U.S. Patent 6,444,447).

Experiments with recombinant PmHAS demonstrated that single sugars are added to the growing chain sequentially; the intrinsic fidelity of each transfer step assures the production of the GAG repeat structure.[3] Another potential mechanism, the simultaneous addition of a disaccharide unit to the nascent chain, does not occur. The other synthases, PmCS, PmHS1 and 2, behave in a similar fashion adding saccharides one at a time.

Thus other possible synthetic reactions include:

$$\text{UDP-GlcA} + [\text{HexNAc-GlcA}]_n \rightarrow \text{UDP} + \text{GlcA-}[\text{HexNAc-GlcA}]_n \qquad \text{(Eq. 2)}$$

$$\text{UDP-HexNAc}+[\text{GlcA-HexNAc}]_n \rightarrow \text{UDP}+\text{HexNAc-}[\text{GlcA-HexNAc}]_n \qquad \text{(Eq. 3)}$$

An interesting feature of the *Pasteurella* GAG synthases is that they appear to elongate the acceptors in a non-processive fashion *in vitro* with chain release between sugar addition steps. As noted later, this property facilitates chemoenzymatic synthesis reactions *in vitro*.

3.1.3 Synthesis of Monodisperse GAG polymers

The *Pasteurella* GAG synthases add sugars onto the non-reducing terminus of an existing GAG chain in a rapid fashion, but if no acceptor is present, the enzymes will spontaneously initiate new chain formation *de novo*. This initiation rate is slower than the elongation rate. The DeAngelis Laboratory found it is possible to accelerate and to synchronize GAG chain production by adding an acceptor molecule to the reaction mixture. The synchronized non-processive polymerization of all chains in concert results in the production of product with low polydispersity (M_W/M_N~1) (**Figure** 7).[95] In nature, GAG polymers (especially HA) are rather polydisperse (M_W/M_N~2-5) populations. Remarkably, polymers of up to 0.5 MDa are made with polydispersity values in the range of ~1.02 and those up to 2 MDa have values of ~1.2. (M_W/M_N = 1 for a monodisperse polymer).

The size of the final GAG polymer is controlled by the stoichiometric ratio of the acceptors to the UDP-sugars [95]. If the same finite amount of precursors is used in two parallel reactions, then a small amount of acceptor spawns a few long chains, while in contrast, a large amount of acceptors results in many short chains. PmHAS and PmCS catalysts are routinely used in synthesis and development of the analogous PmHS1 system is in progress. The availability of defined, uniform polymer preparations is important because in vertebrate biology, the size of the GAG chain (especially for HA) often dictates its effect on cells or tissues.

3.1.4 Synthesis of Defined, Monodisperse Oligosaccharides

The DeAngelis laboratory has developed the chemoenzymatic synthesis of monodisperse GAG oligosaccharides using single-action mutants.[3] Potential medical applications for HA oligosaccharides (n = ~3-10) include killing

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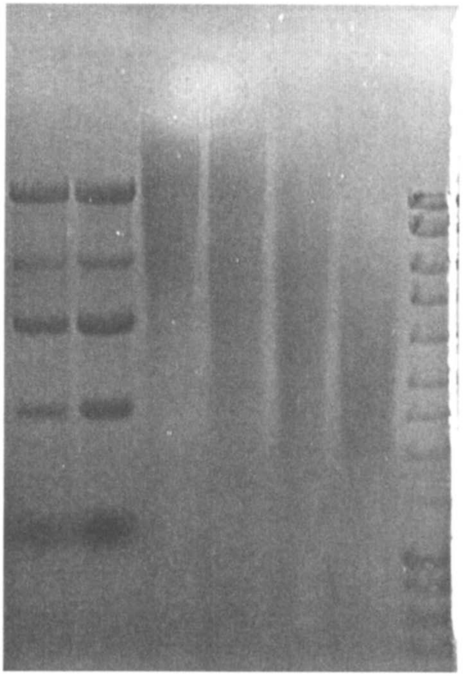

Figure 7. Monodisperse HA preparations versus natural HA and DNA standards.
This agarose gel with Stains-All detection depicts the narrow size distribution of the HA made with synchronized reactions ***(left 2 lanes,*** *a mixture of 5 reactions ranging from 1.5 MDa* ***top*** *to 27 kDa* ***bottom)*** *in comparison to HA from bacteria or chicken* ***(middle 4 lanes).*** *The monodisperse HA bands rival the DNA bands* ***(right lane)*** *which are all composed of a single molecular species.*

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cancerous tumors[96] and enhancing wound vascularization.[97] The *Pasteurella* HA synthase, a polymerizing enzyme that normally elongates HA chains rapidly (~1 to 100 sugars/second) as in Equation 1, was converted by mutagenesis into two single-action glycosyltransferases. The resulting GlcA-transferase and GlcNAc-transferase are appropriate for performing reactions in Equations 2 or 3, respectively. For convenience, soluble forms of mutant pmHAS truncated at the carboxyl termini were purified and immobilized onto beads for utilization as solid-phase catalysts.[3] Similar operations with PmCS create an immobilized GalNAc-transferase. The immobilized enzyme-reactors were used in an alternating fashion to produce quantitatively desirable sugars in a controlled, stepwise fashion without purification of the intermediates (**Figure 8**). The PmHAS and PmCS enzymes are also relatively insensitive to the concomitant UDP byproduct accumulation after many reactor steps. This technology platform is also amenable to the synthesis of tagged (fluorescent-, medicant- or radioactive-labeled) oligosaccharides for biomedical testing.

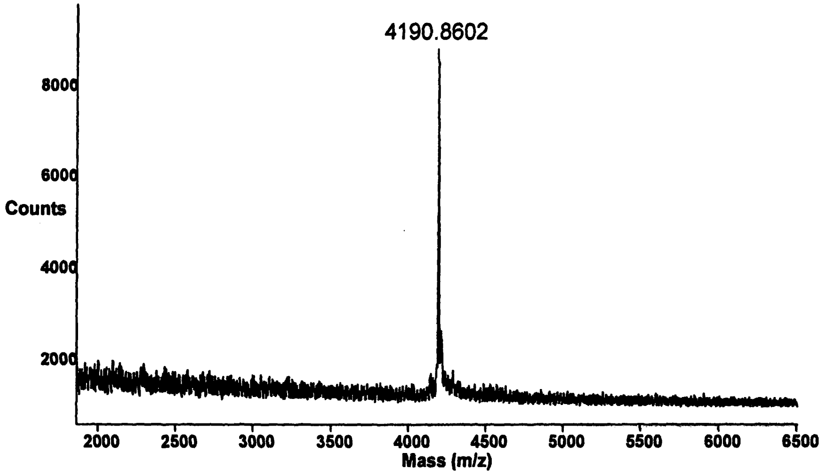

Figure 8. Mass Spectra of HA22 formed with Immobilized Enzyme reactors. *This MALDI-ToF MS shows the product formed by extending a HA4 tetrasaccharide starting sugar with 18 reactor steps (9 with GlcNAc-Tase and 9 GlcA-Tase in alternating fashion). No intermediary purifications were performed before the final desalting step.*

4. GLYCOSAMINOGLYCAN SULFOTRANSFERASES

Glycosaminoglycan sulfotransferases carry out the sulfonation of the polysaccharides provided with various backbone structures to biosynthesize the highly sulfated polysaccharides, glycosaminoglycans (GAGs). The enzymes transfer the sulfo group from a universal sulfate donor, 3'-phosphoadenosine 5'-phosphosulfate (PAPS), to the NH2- or OH positions of the carbohydrate unit of the GAG. Glycosaminoglycan sulfotransferases are Golgi sulfotransferases and include chondroitin sulfate, keratan and heparan sulfate sulfotransferases, which are involved in the biosynthesis of chondroitin sulfate, keratan sulfate, and heparan sulfate, respectively (**Table 4**). The enzymes are highly selective toward the acceptor sites by recognizing the saccharide backbone structures as well as the surrounding detailed saccharide sequences. The recent availability of GAG sulfotransferases has offered additional tools for investigating the relationship between the sulfated saccharide sequences and physiological roles of GAGs and the mechanism of the biosynthesis of GAGs.[58]

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Among various glycosaminoglycans, the biosynthesis of heparan sulfate (HS) has been studied extensively. The biosynthesis of HS occurs in the Golgi apparatus, and is involved in four classes of sulfotransferases: N-deacetylase/N-sulfotransferases (NDST) removes the acetyl group from the N-acetylated glucosamine (GlcNAc) unit and sulfonates the resultant unsubstituted glucosamine unit (GlcNH2) to form N-sulfo glucosamine; 2-O-sulfotransferase (2-OST) sulfonates the 2-OH position of glucouronic acid (GlcA) or iduronic acid (IdoA) units; 6-O-sulfotransferase (6-OST) sulfonates the 6-OH position of N-sulfo- or N-acetylated glucosamine units; and 3-O-sulfotransferase (3-OST) sulfonates the 3-OH position of the N-sulfo or N-unsubstituted glucosamine units. NDST was the first reported HS sulfotransferase.[98] Whether all these enzymes are required to act in a precisely sequential manner to generate the fully modified HS is not completely clear. However, a large amount of evidence demonstrated that the generation of N-sulfo glucosamine unit (by NDST) is the very first modification step followed by epimerization and 2-O-sulfonation.[58]

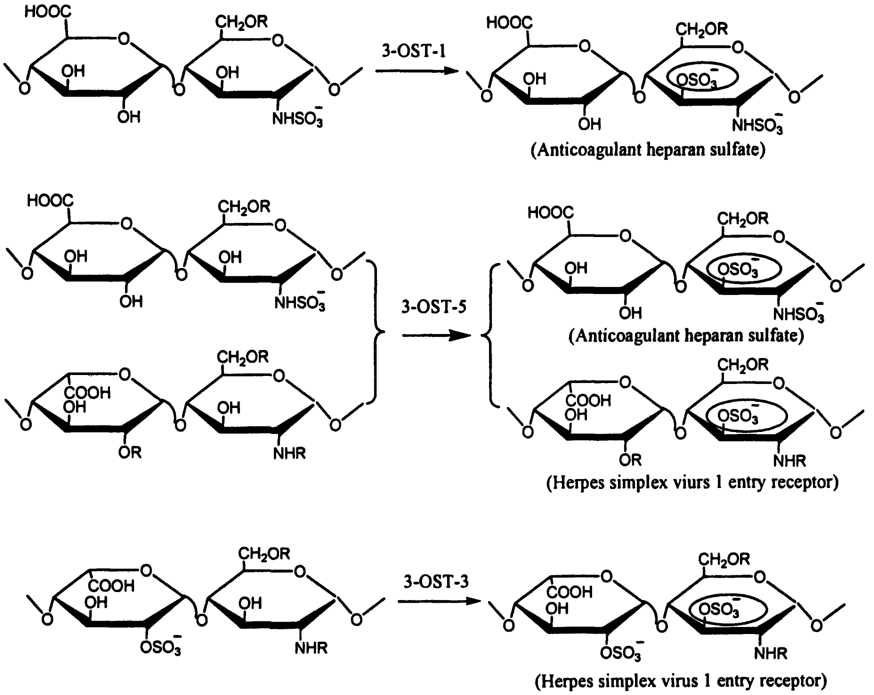
HOOC
CH2OR
O
OH
NHSO3-
3-OST-1
OSO3-
(Anticoagulant heparan sulfate)
COOH
OR
NHR
3-OST-5
(Herpes simplex viurs 1 entry receptor)
3-OST-3
(Herpes simplex virus 1 entry receptor)

Figure 9. The substrate specificities of 3-OST-1, 3-OST-3, and 3-OST-5. *3-OST-1 sulfonates the glucosamine unit that is linked to a nonsulfonated glucuronic acid at the nonreducing end; 3-OST-3 sulfonates a glucosamine unit that is linked to 2-O-sulfo iduronic acid at the nonreducing end; and 3-OST-5 sulfonates both type of disaccharides. R represents* $-H$ *or* $-SO_3H$.

Table 4. Glycosaminolgycan sulfotransferases

Enzyme	Substrate specificity
Heparan sulfate sulfotransferases	
N-deacetylase/*N*-sulfotransferase[98, 99]	Removal of the acetyl group and sulfation of NH position of GlcNAc
2-*O*-sulfotransferase[100]	Sulfation of 2-OH position of GlcA or IdoA
6-*O*-sulfotransferase[101, 102]	Sulfation of 6-OH position of GlcNS or GlcNAc
3-*O*-sulfotransferase[103]	Sulfation of 3-OH position of GlcNS or $GlcNH_2$
Keratan sulfotransferase	
Keratan sulfate Gal-6-sulfotransferase[104]	Sulfation of 6-OH position of Gal
Chondroitin sulfate sulfotransferase	
Chondroitin 4-sulfotransferase[105]	Sulfation of 4-OH position of GalNAc
Chondroitin 6-sulfotransferase[106]	Sulfation of 6-OH position of GalNAc
GalNAc ($4SO_4$) 6-sulfotransferase[107]	Sulfation of 6-OH position of GalNAc4S
Uronosyl 2-*O*-sulfotransferase[108]	Sulfation of 2-OH of GlcA or IdoA

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All HS sulfotransferases, with the exception of 2-OST, are present in multiple isoforms. These isoforms are highly homologous in amino acid sequences and expressed at distinct levels among different tissues, probably playing an important role in biosynthesizing tissue-specific HS.[109] For example, NDST is present in four different isoforms, 6-OST is present in three isoforms,and 3-OST is present in seven isoforms. The unique substrate specificities of different 3-OST isoforms have been reported, demonstrating that different isoforms recognize the saccharide residues at the nonreducing end of the acceptor site (**Figure 9**). Very interestingly, the HS modified by different 3-OST isoforms exhibit distinct physiologic/pathophysiologic functions. HS modified by 3-OST-1 binds to antithrombin, carrying anticoagulant activity [110], whereas HS modified by 3-OST-3 binds to herpes simplex virus glycoprotein D and serves as an entry receptor for herpes simplex virus 1.[111] HS modified by 3-OST-5 exhibit both the anticoagulant activity and serves as an entry receptor for herpes simplex virus 1 entry receptor.[112] NDST has four different isoforms.[98, 99, 113, 114] It is believed that these isoforms are specialized in generating specific N-sulfo glucosamine clusters and control relative ratio of GlcNH2/GlcNAc/GlcNS.[99] The substrate specificities among 6-OST isoforms are not experimentally distinguishable.[115, 116] The substrate subtleties among the isoforms allow the synthesis of unique sulfonated saccharide sequences.

4.1. Structure of HS sulfotransferases

There is considerable interest in understanding the mechanism used by the sulfotransferases to recognize their substrates. Furthermore, understanding this mechanism could aid the design of specific GAG sulfotransferase inhibitors to manipulate the biosynthesis of the unique subtypes of sulfonated saccharide structures.[117, 118] Among GAG sulfotransferases, only three crystal structures of HS sulfotransferases are available, including NST-1, 3-OST-1 and 3-OST-3.[119-121] NST-1 is the *N*-sulfotransferase domain of NDST isoform -1, which transfers the sulfo group to the *N*-position of glucosamine unit. All three enzymes show a very similar overall folded structure, consisting of a five-stranded parallel β-sheet flanked on both sides by α-helices.[122] The structural features of HS binding sites are distinct between NST-1 and 3-OST-1, supported by the fact NST-1 sulfonates the polysaccharide with low or no sulfation, whereas 3-OST-1 sulfonates the polysaccharide with high level of sulfation. The structures of the active sites of 3-OST-1 and 3-OST-3 are very similar. Based upon the crystal structures and the results of mutagenesis and computational analyses, the role of several amino acid residues in catalyzing the sulfotransferase activity were identified.[120, 123, 124] A unique hydrogen bond network involved in key catalytic residues was discovered in 3-OST-1 and 3-OST-3, but not in NST-1, suggesting that this network contributes to the 3-*O*-sulfotransferase activity.[120, 121]

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The best available example to understand how the sulfotransferases interact with their substrates is from the study of the structure of a ternary complex of 3-OST-3/PAP/tetrasaccharide (where PAP represents 3'-phosphoadenosine 5'-phosphate). From this structure, one can clearly observe the interaction between the amino acid residues that participate in the binding to the substrate.[121] The 3-OH position of the glucosamine unit (acceptor site) is locked into a position that is about 2.8 Å to the catalytic base residue. At least six amino acid residues interact with the different functional groups of the saccharide units around the reducing end and the nonreducing end of the glucosamine acceptor (**Figure 10**).

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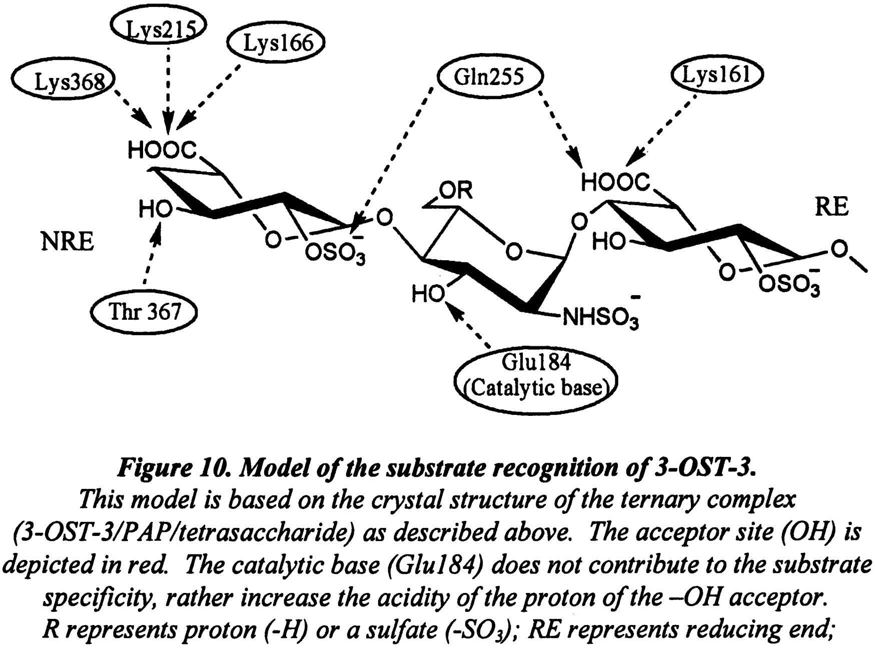

Figure 10. Model of the substrate recognition of 3-OST-3.
This model is based on the crystal structure of the ternary complex (3-OST-3/PAP/tetrasaccharide) as described above. The acceptor site (OH) is depicted in red. The catalytic base (Glu184) does not contribute to the substrate specificity, rather increase the acidity of the proton of the –OH acceptor. R represents proton (-H) or a sulfate (-SO$_3$); RE represents reducing end; NRE represents non-reducing end. The three ends at the NRE of the tetrasaccharide are shown.

In addition, the iduronic and uronic acid units of the tetrasaccharide substrate are present in a skew-boat conformation when the tetraccharide binds to 3-OST-3, whereas, these two units are present in a chair conformation when this tetrasaccharide binds to fibroblast growth factor 2.[15] This observation suggests that the conformations of the saccharide units might contribute to the binding recognition between enzymes and substrates. Using site-directed mutagenesis approach, two amino acid residues (Q255 and K368) of 3-OST-3 were identified to participate in binding the substrate during the catalysis. Mutation of Q255 and K368 led to the significant loss of 3-OST-3 activity, whereas mutations of similar amino acid residues of 3-OST-1 have no effect on the enzymatic activity. It was also reported that 3-OST-1 undergoes a conformational change when it binds to the substrate, suggesting that protein flexibility or potential allosteric effects on the substrate recognition.[125] The contribution of the conformational changes of 3-OST-1 to the substrate specificity requires further investigation.

4.2 Synthesis of HS using HS biosynthetic enzymes

Chemical synthesis has been the major route to obtain structurally defined heparin and HS oligosaccharides.[126] The most important example involves the structure of AT-binding pentasaccharide (Arixtra).[127] As described above, Arixtra is a specific factor Xa inhibitor. Unfortunately, the total synthesis of heparin and HS oligosaccharides, larger than pentasaccharides, is extremely difficult. HS analogs with 14 or 16 saccharide units inhibit the activity of thrombin, but these compounds are simplified hybrid molecules of HS oligosaccharides and highly sulfonated glucose units and are not the naturally occurring structures.[54] While a number of groups continue to pursue the synthesis of heparin,[128] it has become clear that chemical synthesis alone will be incapable of generating most larger oligosaccharide structures. The application of HS biosynthetic enzymes for generating large heparin and HS oligosaccharides with desired biological activities offers a promising alternative approach.

The enzymatic synthesis includes one-step enzymatic modification or multi-step modification as described below. One step enzymatic modification to synthesize biologically active oligosaccharides or polysaccharide saccharides is straightforward. For example, 2-OST was used to modify HS oligosaccharide libraries to synthesize FGF and FGF receptor binding domains of HS.[115, 129] Likewise, a herpes simplex virus glycoprotein D-binding domain was isolated from 3-OST-3-modified HS oligosaccharide library.[130] However, this approach is limited by the availability of the oligosaccharide library. Furthermore, the subsequent purification is often difficult.

The multi-step enzymatic synthesis of the antithrombin-binding pentasaccharide from E. coli capsular polysaccharide was reported by Kuberan and colleagues (**Figure 11**).[60] This approach demonstrated for the first time the feasibility of enzymatic synthesis of a HS oligosaccharide with defined structure and anticoagulant polysaccharides. Unfortunately, only microgram amounts of products were generated, making a general approach for extensive biological studies impossible. Recently, Lindahl and colleagues reported an alternative chemoenzymatic approach for the synthesis of anticoagulant heparin from heparosan, the E. coli K5 capsular polysaccharide.[131] This method utilized the C5 epimerase to convert D-glucuronic acid (GlcA) to L-iduronic acid (IdoA), followed by the chemical persulfonation and finally selective desulfonation.

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While this approach afforded gram quantities of a heparin-like polysaccharide with anticoagulant activity, unnatural saccharide units, such as 3-O-sulfo-D-glucuronic acid, were present in their product, suggesting a limitation in the selectivity of chemical sulfonation/desulfonation in HS synthesis.

Two obstacles remain in improving the scale the enzymatic synthesis of HS: the availability of large amount of HS sulfotransferases and the inhibition effect of 5'-phosphoadenosine 3'-phosphate (PAP), the desulfated product of PAPS. Liu and Linhardt Labs have recently developed an approach to enable the scale of the enzyme-catalyzed O-sulfations using completely desulfated and N-sulfated heparin (**9**) as a starting material (**Figure 12**).[132]

First, highly active HS O-sulfotransferases were expressed in *E.* coli, allowing the access of large amount of sulfotransferases. Second, the sulfotransfer reactions were coupled with the PAPS regeneration system that was developed by the Wong group,[133] eliminating the inhibition effect of PAP. The PAPS regeneration system utilizes arylsulfotransferase IV, which can converts PAP to PAPS as illustrated in **Figure 12B**. In addition, the PAPS regeneration system permits the use of the p-nitrophenol sulfate PNPS as the sulfo donor and requires only catalytic amounts of PAP, significantly reducing the cost of synthesis.[133] The immobilized enzymes used in this approach are capable of reuse and the HS sulfotransferases show improved thermal stability, suggesting that this approach can be easily expanded for a large scale synthesis. This method generates the milligram scale of sulfated polysaccharide with the desired biological activities, including the anticoagulant activity as measured by the anti-Xa and anti-IIa activities (compound **13**), the activity in triggering FGF/FGF receptor signaling (compounds **10**, **11**, **12a** and **12b**) and the binding to herpes simplex virus glycoprotein D (compound **14**).

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5. CONCLUSIONS

Recent advances in carbohydrate chemistry have made GAG oligosaccharides more accessible while still posing a significant synthetic challenge. The enzymes involved in GAG biosynthesis and metabolism play crucial role in understanding the structures of these heteregenous polysaccharides as well as preparing structurally defined GAGs. The use of three GAG modifying enzymes, lyases, synthases and sulfotransferases, have been applied to the preparation of biologically potent, structurally defined GAG oligosaccharides and polysaccharides.

Polysaccharide lyases can be combined with separation methods such as chromatography and electrophoresis for the preparation of glycosaminoglycan oligosaccharides for biological evaluations as well as for disaccharide analysis, oligosaccharide mapping and polysaccharide sequencing.

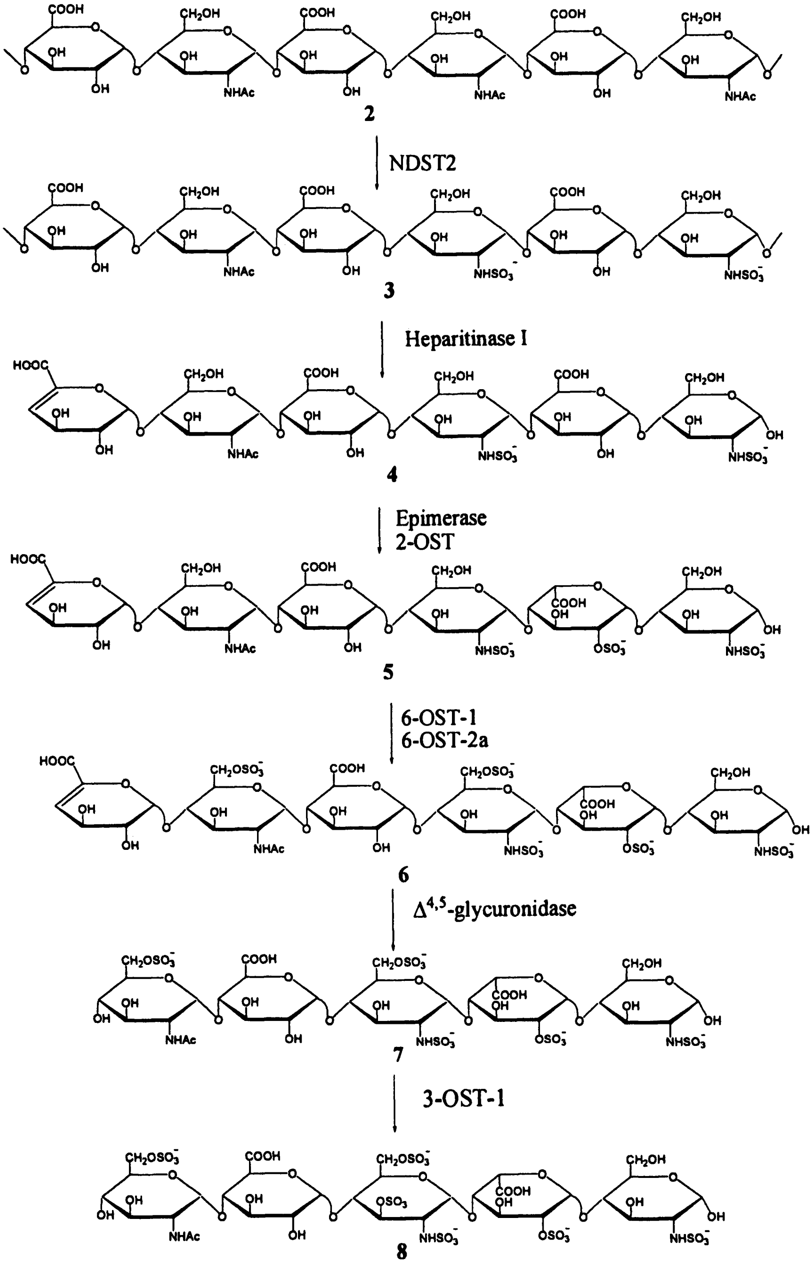
2
NDST2
3
Heparitinase I
4
Epimerase
2-OST
5
6-OST-1
6-OST-2a
6
Δ4,5-glycuronidase
7
3-OST-1
8

Figure 11. Synthesis of antithrombin-binding pentasaccharide from E. coli K5 capsular polysaccharide.

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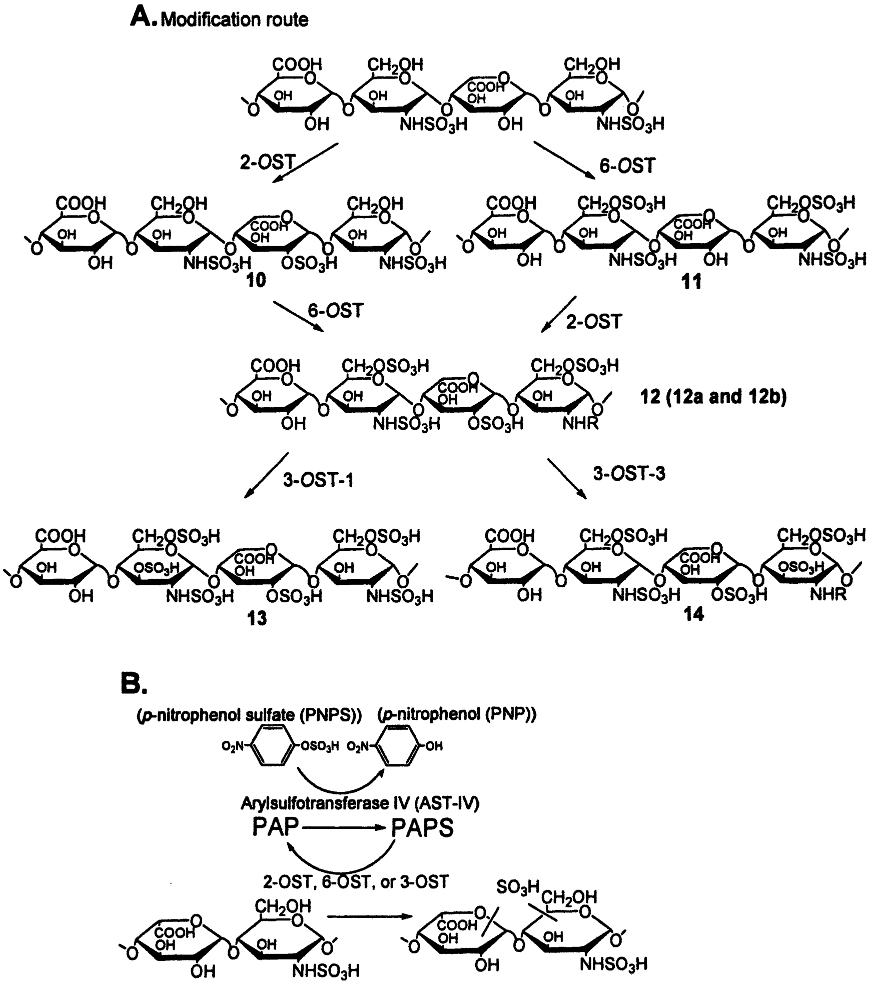
A. Modification route
2-OST
6-OST
10
11
6-OST
2-OST
12 (12a and 12b)
3-OST-1
3-OST-3
13
14
B.
(p-nitrophenol sulfate (PNPS))
(p-nitrophenol (PNP))
Arylsulfotransferase IV (AST-IV)
PAP
PAPS
2-OST, 6-OST, or 3-OST

Figure 12. Schematic synthesis of sulfonated polysaccharides and PAPS regeneration system.

*Panel A shows the stepwise enzymatic synthesis of sulfonated polysaccharides using HS sulfotransferases. The description of intermediate polysaccharides is shown in the text. Compound **12a** and **12b** were prepared by inverting the order of sulfonation steps. **12a** was prepared by incubating compound **9** with 2-OST followed by 6-OST, while **12b** was prepared by incubating compound **9** with 6-OST followed by 2-OST. Panel B shows the reaction catalyzed by arylsulfotransferase IV (AST-IV) to generate PAPS. R = -H or* $-SO_3$.

Versatile, malleable *Pasteurella* synthases have been harnessed as useful catalysts for the creation of a variety of defined GAG or GAG-like polymers ranging in size from small oligosaccharides to huge polysaccharides. These materials should be useful for a wide spectrum of potential biomedical products for use in the areas of cancer, coagulation, infection, tissue engineering, drug delivery, surgery, and viscoelastic supplementation.

GAG sulfotransferases play critical roles in biosynthesizing the GAGs with diversified biological functions. The availability of the GAG sulfotransferases not only leads to an improved understanding of the structure-function relationship of the sulfated polysaccharides but also opens new routes to synthesize these molecules. Although the current attention has been focused on HS and heparin synthesis, it is conceivable that a similar enzymatic approach could be used for the synthesis of other GAGs, including chondroitin sulfates and keratin sulfates. A further simplified synthetic approach could lead to a general method to prepare "recombinant" glycosaminoglycans, in which will be possible to use glycomics approach to study the effects in different biological systems.

The use of these three enzyme families in combination should greatly enhance the success of target GAG synthesis. The lyase prepared oligosaccharides could be used as templates to be elongated with UDP-monosaccharide donors using synthases then the sulfotransferases could introduce the sulfo groups into the specific positions leading to the synthesis of desired GAG structures. In summary, expansion of the enzymatic synthesis, in combination with chemical synthesis, will lead to novel therapeutic reagents in physiological and pathophysiological events.

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Chapter 16

The Chemical Synthesis of Glycosylphosphatidylinositol Anchors from *Trypanosoma cruzi* Trypomastigote Mucins

Exploration of Ester and Acetal Type Permanent Protecting Groups

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Abstract: The protozoan parasite *Trypanosoma cruzi* is a causative agent of Chagas' disease, which affects millions of people in South and Central America. Throughout the life cycle, *T. cruzi* produce both common and stage-specific glycosylphosphatidylinositol(GPI)-anchored cell-surface macromolecules. The purified GPI fraction of *T. cruzi* trypomastigote mucins (tGPI) revealed extraordinary pro-inflammatory activity (comparable to those of bacterial lipopolysaccharide), which was allegedly associated with the presence of unsaturated fatty acids in the lipid moiety (structures **1** and **2**). Two distinct novel synthetic strategies, both implicating the use of non-benzyl type protecting groups, were designed and applied for the chemical preparation of the tGPIs **1** and **2**.

NOTE: This chapter is dedicated to professor Nikolay K. Kochetkov (Zelinsky Institute of Organic Chemistry, Moscow, Russia) on the occasion of his 90th birthday.

Introduction

Glycosylphosphatidylinositols (GPIs) are a class of natural glycosylphospholipids that anchor proteins and glycoproteins (via their *C*-terminus) as well as phosphoglycans (via the reducing end of the chain) to the membrane of eukaryotic cells. Since the first full assignment of a GPI structure in 1988,[1] quite a few GPI anchors were characterised.[2] The function of the compounds (in addition to the clear one of linking the above biopolymers to membranes) has been extensively discussed[2,3] and there is also evidence that GPIs and/or metabolites of them can act as secondary messengers, modulating biological events including insulin production, insulin-mediated signal transduction, cellular proliferation and cell-cell recognition. The discovered role as mediators of regulatory processes makes the chemical preparation of the compounds and their analogues of great interest. Up to date, a number of syntheses of GPIs (yeast,[4] rat brain Thy-1,[5] *T. brucei*,[6] *Leishmania*,[7] *T. gondii*,[8] *P. falciparum*[9] and *T. cruzi* IG7 antigen[10]) have been reported.

The protozoan parasite *Trypanosoma cruzi* is a causative agent of Chagas' disease, which affects millions of people in South and Central America. The WHO estimations show 18 million individuals are currently infected and 95 million are at risk in 19 endemic countries. Chagas' disease makes 2.8 million people disabled annually.[11] It is transmitted to mammals in faeces of biting insect vector (haematophagous triatomine bug) and has four distinct developmental stages. Throughout the life-cycle, *T. cruzi* produce both common and stage-specific GPI-linked cell-surface macromolecules.[12-15] Local release of GPI-anchored mucins by the bloodstream trypomastigote stage of the parasite is believed to be responsible for development of parasite-elicited inflammation causing cardiac and other pathologies associated with acute and chronic phases of Chagas' disease.[15]

The trypomastigote GPI-anchored mucins (tGPI mucins) appear to be a double-dealing substance. After getting into the bloodstream, the parasite must limit its own success in the body, so as to prolong the life of its host. Aiming this, it produces the tGPI mucins, which, first, suppress the infection. Unfortunately, in about one in five patients, prolonged exposure to the same substance leads to the life-threatening conditions of chronic Chagas' disease (i.e., irreversible damage to the heart and/or digestive tract).[15] It has been suggested[16] that **Charles Darwin** acquired the disease during his historic voyage on *The Beagle* and that it was the chronic form that turned him into an invalid for 40 years of his life after his return home and ultimately was responsible for his death in 1882.

It's been recently[13] discovered that purified GPI fraction of *T. cruzi* trypomastigote mucins (i.e., tGPI) revealed extraordinary pro-inflammatory activities, comparable to those of bacterial lipopolysaccharide. Being able to trigger induction of tumor necrosis factor-α, interleukin-12 and nitric oxide at 2-30 pM level (when presented to macrophages), tGPI proved to be one of the most potent microbial pro-inflammatory agents known. The structure of the cytokine- and NO-inducing tGPI anchor has been defined[13] and the extreme biological activity was putatively associated with the presence of unsaturated fatty acids in the *sn*-2 position of

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alkylacylglycerophosphate moiety(*) (see structures **1** and **2** on Scheme 1) and/or D-galactose branches along the glycan core (non-stoichiometric, not shown). Since the issues regarding the structural features responsible for the activity can only be resolved through the synthesis, a multidisciplinary programme has been launched in this laboratory aimed at the chemical preparation of various *T. cruzi* trypomastigote mucin GPIs (including also those containing D-galactose branches) and the meticulous elucidation of their structure-activity relationships. Now we report the first chemical syntheses of tGPIs from *T. cruzi* bearing oleic (compound **1**) and linoleic (compound **2**) acid moieties.

Results and Discussion

There are two major structural features that differ compounds **1** and **2** from the GPIs synthesised previously:[4-10] (i) the presence of unsaturated fatty acids in the lipid moiety instead of saturated ones and (ii) the existence of 2-aminoethylphosphonate at O-6 of D-glucosamine moiety, which is a parasite specific substituent for *T. cruzi* only (this position is un-substituted in GPIs from other species). Since the presence of double bonds was not compatible with the use of benzyl ethers (widely used before[4-10]) as permanent *O*-protecting groups for assembly of protected GPI derivatives, novel synthetic strategies were explored. Strategy **A** (Scheme 1) was designed to imply mild base labile (esters) and acid labile (acetals and *N*-Boc) permanent protecting groups, whereas strategy **B** (Scheme 7) suggested the use of acid labile (diacetals, acetals and *N*-Boc) and fluoride ion labile (*tert*-butyldimethylsilyl ethers, or TBS ethers) groups for this purpose.

Synthetic strategy A

The first strategy exploited benzoic esters, acetals and *N*-Boc groups for permanent *O,N*-protection, while various silyl ethers were employed as orthogonal blocking groups for O-6 of D-glucosamine (TES), O-6 of D-mannose-3 (primary TBS) and O-1 of *myo*-inositol (secondary TBS) to ensure further introduction of P-containing esters. For the final deprotection, we anticipated no problem with acidic cleavage of acetals and *N*-Boc groups, but expected that mild basic treatment in polar solvent would preferentially cleave the benzoates and leave the fatty ester of the lipid mostly intact because of the micelle formation. Following the retro-synthetic disconnection on Scheme 1, the GPIs **1** and **2** were assembled from the mannotetraose building block **5** and the azidoglucose-inositol block **6**. The phosphonodichloridate **3** and the H- phosphonates **4** and **7** (or **8**) were used for 'P-decoration', i.e., sequential introduction of the 2-aminoethylphosphonate, ethanolamine phosphate and acylalkyl-glycerophosphate fragments, respectively.

(*) Content of fatty acid components in the biologically active tGPI anchor fraction was found to be: oleic acid (C18:1, 31%), linoleic acid (C18:2, 21%) and palmitic acid (C16:0, 37%).[13]

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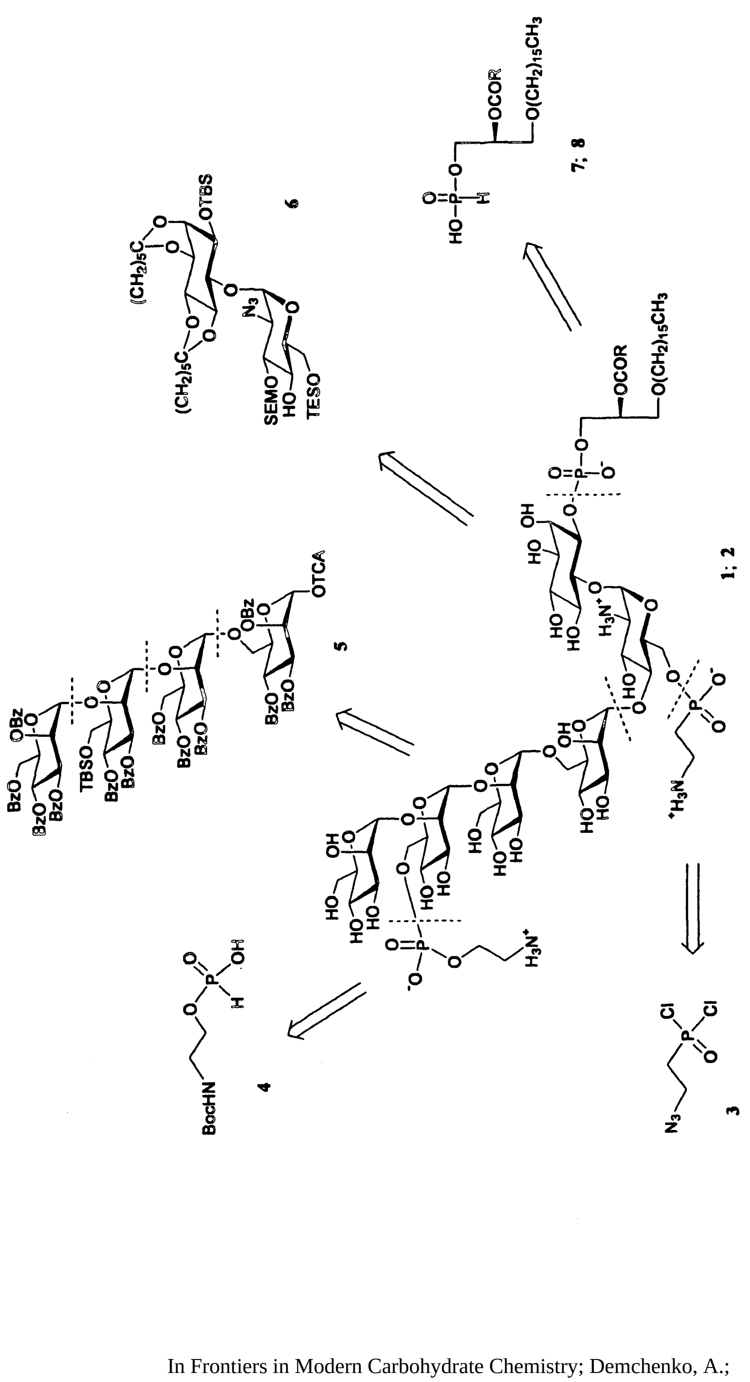
BocHN
4
3
5
6
1; 2
7; 8
TBSO
SEMO
HO
TESO
N3
OTBS
OTCA
OCOR
O(CH2)15CH3
H3N+
+H3N

1 and 7 R = $(CH_2)_7CH{=}CH(CH_2)_7CH_3$; 2 and 8 R = $(CH_2)_7CH{=}CHCH_2CH{=}CH(CH_2)_4CH_3$

Scheme 1. Retrosynthetic scheme A. *Boc* = tert-*BuOC(O), SEM* = *$Me_3Si(CH_2)_2OCH_2$, TBS* = tert-*$BuMe_2Si$, TCA* = *$C(NH)CCl_3$, TES* = *Et_3Si.*

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The tetrasaccharide block **5** was prepared from the monosaccharide derivatives **9-12**, which were assembled on step-by-step manner as presented on Scheme 2. Compounds **9** and **12**, in turn, were made from D-mannose (in 85 and 68% yield, respectively) in three steps each using conventional methods. The preparation of the derivatives **10** and **11** progressed also from D-mannose (in 40 and 68% yield, respectively) via the common intermediate, known 3,4,6-tri-*O*-acetyl-1,2-*O*-(1-methoxyethylidene)-β-D-mannopyranose,[17] which was a subject of further protecting group re-modelling. Thus, standard deacetylation (with MeONa in MeOH) and benzoylation followed by opening of the ortho-ester (with aq. TFA), acetylation, anomeric deprotection (with Me_2NH in THF) and treatment with CCl_3CN in the presence of Cs_2CO_3 gave the trichloroacetimidate **11**. In the synthesis of **10**, 1,2-*O*-(1-methoxyethylidene)-β-D-mannopyranose was successively treated with TBSCl and then BzCl in pyridine, aq. TFA and Ac_2O in pyridine to form 1,2-di-*O*-acetyl-3,4-di-*O*-benzoyl-6-*O*-*tert*-butyldimethylsilyl-D-mannopyranose. Subsequent desilylation (TBAF), benzylation with benzyl trichloroacetimidate and triflic acid, anomeric deacetylation (Me_2NH) and the reaction with CCl_3CN and Cs_2CO_3 gave the trichloroacetimidate **10**.

For the oligosaccharide chain elongation, the disaccharide **13** (94%) was made first by coupling of the glycosyl acceptor **12** and the trichloroacetimidate **11** in the presence of trimethylsilyl triflate (TMSOTf). It was then deacetylated[18] with HCl in MeOH (→**14**) followed by the reaction with glycosyl donor **10** and TMSOTf to produce the trisaccharide **15** (96%). Subsequent deacetylation (→**16**) and one more glycosylation with the trichloroacetimidate **9** and TMSOTf provided the tetrasaccharide **17** (99%). Compound **17** was then converted to the mannotetraose glycosyl donor **5** (in 66% yield) by consecutive re-protection at O-6" (hydrogenation over Pd catalyst followed by silylation with TBSOTf/Et_3N; →**18**), anomeric debenzoylation with ethylenediamine and the reaction with CCl_3CN in the presence of Cs_2CO_3.

The azidoglucose-inositol block **6** was synthesised (Scheme 3) from the pseudo-disaccharide **23**, which was described by R. R. Schmidt[4,19] as a glycosylation product of the optically pure D-*myo*-inositol derivative **21**[20] with the azidoglucose trichloroacetimidate **19**. We found one could avoid the resolution of diastereoisomeric (–)-menthyloxycarbonyl-*myo*-inositols **21** (D-product) and **20** (L-product), but to perform glycosylation of the whole mixture and then isolate the required derivative **23** by standard flash column chromatography (SiO_2). Coupling of the mixture **21**+**20** (7:3, according to ^1H NMR data; prepared as described in Ref. 20) with the glycosyl donor **19**[21] in the presence of TMSOTf and molecular sieves 4Å proceeded smoothly and gave the easily separable (R_f difference of 0.1) diastereoisomers **23** and **22**, which were isolated in 64 and 30% yield, respectively (i.e., 94% total yield of the glycosylation based on the mixture **21**+**20**). Both compounds had α-configuration of the D-glucoside bond, which was confirmed by the characteristic values (3.5 Hz for **23** and 3.6 Hz for **22**) for $J_{1',2'}$ coupling constants. Clearly, 2-azido-2-glucose-α-D-glucosyl moiety worked as additional powerful chiral auxiliary and facilitated the separation of D- and L-*myo*-inositol derivatives.

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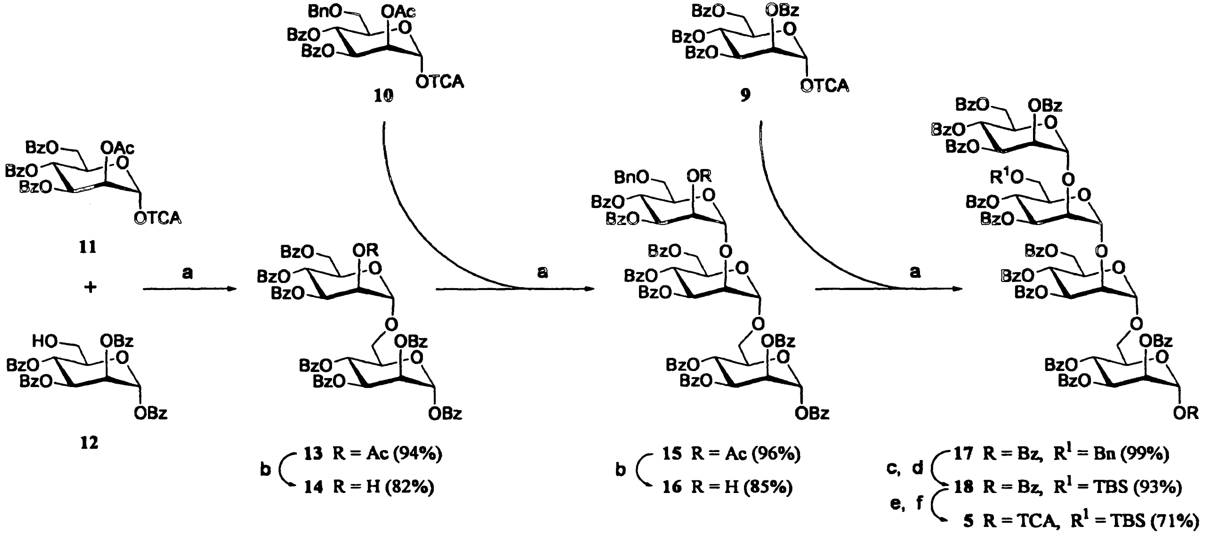
BnO
OAc
BzO
BzO
O
OTCA
10
BzO
OBz
BzO
BzO
O
OTCA
9
BzO
OAc
BzO
BzO
O
OTCA
11
+
HO
OBz
BzO
BzO
O
OBz
12
a
OR
OBz
13 R = Ac (94%)
14 R = H (82%)
b
a
BnO
15 R = Ac (96%)
16 R = H (85%)
b
a
R¹O
OR
17 R = Bz, R¹ = Bn (99%)
18 R = Bz, R¹ = TBS (93%)
5 R = TCA, R¹ = TBS (71%)
c, d
e, f

Scheme 2. Reagents: *a) TMSOSO$_2$CF$_3$, DCM; b) 2% HCl, MeOH/DCM; c) H$_2$, Pd(OH)$_2$/C, THF; d) TBSOSO$_2$CF$_3$, Et$_3$N, DCM; e) NH$_2$CH$_2$CH$_2$NH$_2$·HOAc, THF; f) CCl$_3$CN, Cs$_2$CO$_3$, DCM.*

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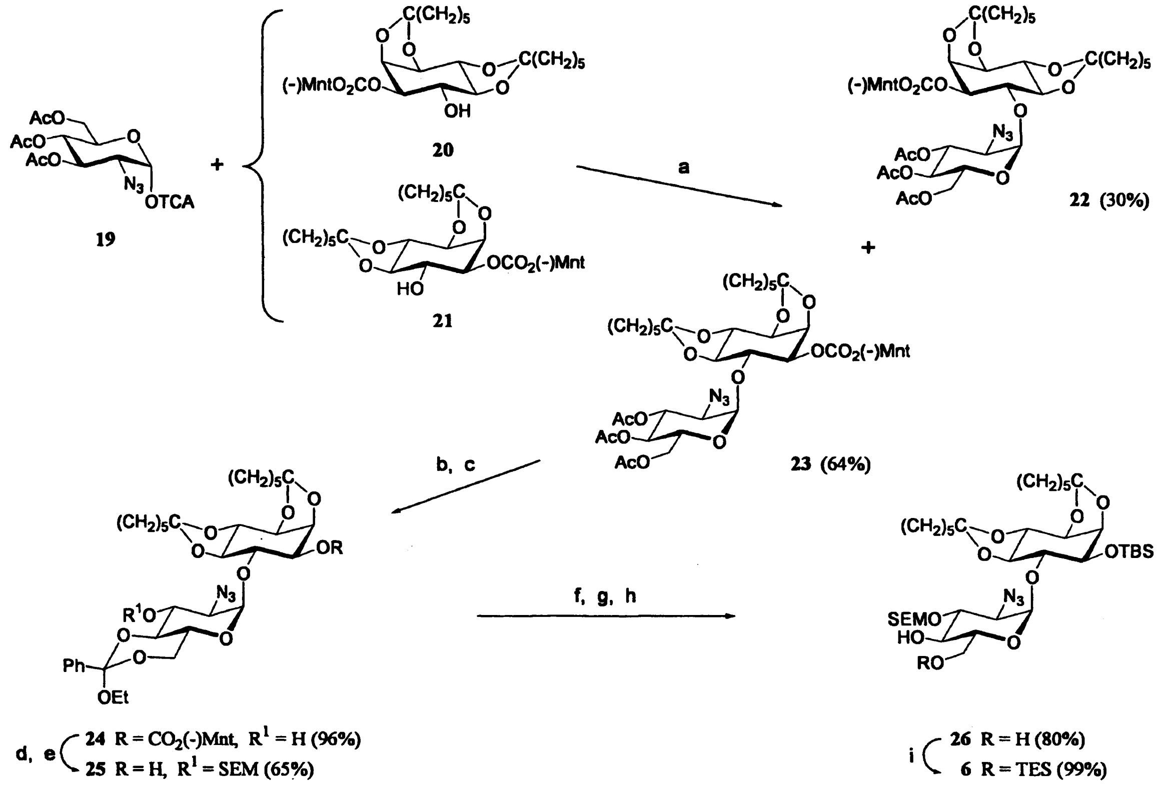
19
20
21
22 (30%)
23 (64%)
a
b, c
f, g, h
d, e
24 R = CO2(-)Mnt, R1 = H (96%)
25 R = H, R1 = SEM (65%)
i
26 R = H (80%)
6 R = TES (99%)
AcO
N3
OTCA
(-)MntO2CO
C(CH2)5
OH
HO
OCO2(-)Mnt
OR
R1O
Ph
OEt
SEMO
RO
OTBS

Scheme 3. Reagents: *a) TMSOSO$_2$CF$_3$, MS 4Å, Et$_2$O/DCM; b) 0.02 M NaOMe, MeOH/DCM; c) PhC(OEt)$_3$, camphor-10-sulfonic acid, DCM; d) SEMCl,* i*-Pr$_2$NEt, DCM; e) 1 M NaOMe, MeOH/DCM; f) TBSOSO$_2$CF$_3$, Et$_3$N, DCM; g) DCM-CF$_3$COOH-water (1000:1:0.1); h) 0.1 M NaOMe, MeOH/DCM; i) TESCl, pyridine, DCM, (-20 °C). Mnt = menthyl.*

For the transformation **23**→**6**, first, the introduction of the acid labile 2-trimethylsilylethoxymethyl (SEM) permanent protecting group at O-3' was needed. This was performed through the mild basic deacetylation of **23** followed by ortho-esterification with $PhC(OEt)_3$ in mild acidic conditions (to form the 4',6'-ortho-ester derivative **24**) and the reaction with SEM-chloride in the presence of *N*,*N*-di-isopropylethylamine. Following basic cleavage of the (–)-menthylcarbonate gave the 1-OH-pseudodisaccharide **25**, which was then successively silylated with $TBSOTf/Et_3N$, hydrolysed with 0.1% TFA/water in DCM (10 min) to open the ortho-ester (forming a mixture of 4'- and 6'-acetates), deacetylated (with MeONa in MeOH; →**26**) and silylated at O-6' with Et_3SiCl in pyridine-DCM. Thus, the azidoglucose-inositol block **6** was prepared from compound **23** in 7 steps in ~50% overall yield.

The 2-azidoethylphosphonodichloridate **3** was prepared (Scheme 4) from diethyl 2-bromoethylphosphonate **27** via the azidation reaction with NaN_3 (→**28**, $\delta_P = 27.0$) followed by de-esterification with Me_3SiBr and chlorination with oxalyl chloride in the presence of *N*,*N*-dimethylformamide. The 2-(*N*-Boc)-aminoethyl hydrogenphosphonate **4** ($\delta_P = 5.2$, $^1J_{P,H} = 620.1$ Hz) was made by the reaction of *N*-Boc-ethanolamine **29** with salicylchlorophosphosphite[22] followed by hydrolysis. The 2-*O*-acyl-1-*O*-hexadecyl-*sn*-glyceryl hydrogenphosphonates **7** ($\delta_P = 4.6$, $^1J_{P,H} =$ 627.3 Hz) and **8** ($\delta_P = 4.4$, $^1J_{P,H} = 627.3$ Hz) were synthesised starting from commercially available 2,3-*O*-isopropylidene-*sn*-glycerol **30**, which was, first, alkylated with *n*-hexadecyl iodide in the presence of NaH followed by acid hydrolysis to produce 1-*O*-hexadecyl-*sn*-glycerol **31** (55%). It was then successively silylated at 3-OH with Et_3SiCl in pyridine, esterified with oleoyl chloride or linoleoyl chloride and desilylated with $3HF{\cdot}Et_3N$, thus providing the 2-*O*-acylated glycerol derivatives **32** and **33**, respectively. Each of them was converted (almost quantitatively) to the corresponding H-phosphonate derivative (**7** and **8**, respectively) by the reaction with tri-imidazolylphosphine[23] followed by hydrolysis.

With all the principal building blocks in hand, we pursued the preparation of the targeted GPIs **1** and **2**. First, the glycan-inositol backbone **34** was made (Scheme 5) by the glycosylation of the glycosyl acceptor **6** with the mannotetraose trichloroacetimidate **5** in the presence of TMSOTf and molecular sieves 4Å. Subsequent cleavage of the TES group (the 'weakest' of the three silyl protecting groups) with acetic acid buffered TBAF smoothly gave the 6'-OH pseudohexasaccharide derivative **35** (60% from **5**), ready to turn to the 'P-decoration' procedures. 1*H*-Tetrazole assisted esterification of **35** with the phosphonodichloridate **3** followed by methanolysis afforded the phosphonic diester **36**[30] (79%) as a diastereomeric mixture(*) ($\delta_P = 28.5, 28.8$). It was then a subject of successive reduction of the azido groups with Ph_3P, *N*-protection with Boc-anhydride (→**37**)[30] and selective cleavage of the primary TBS ether with $3HF{\cdot}Et_3N$, thus

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(*) The P-protection was required at this stage to avoid undesired modifications of the phosphonate moiety during further transformations. Each of the methyl phosphonate derivatives **36-42** was formed as a mixture of diastereomers at the phosphorus (in a ratio of 1:1), as clearly indicated by ^{31}P NMR ($CDCl_3$) spectra.

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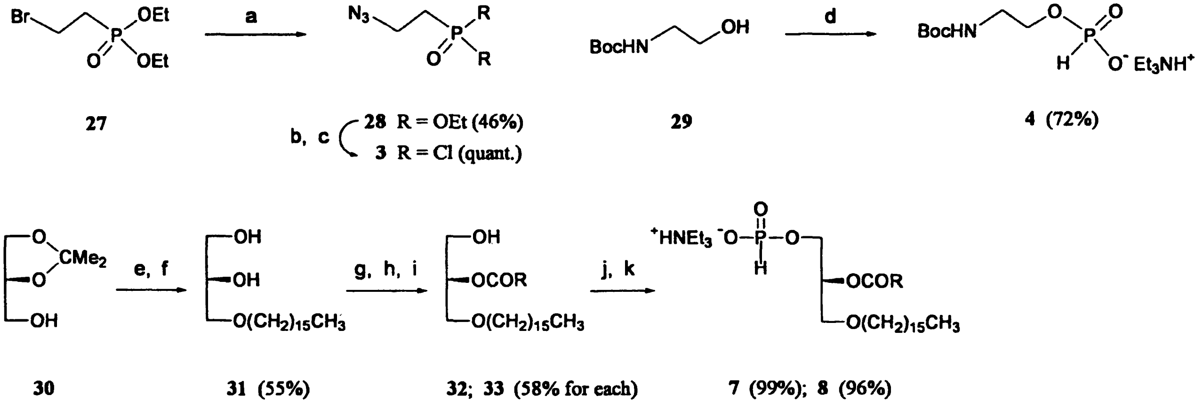
a
b, c
d
e, f
g, h, i
j, k
27
28 R = OEt (46%)
3 R = Cl (quant.)
29
4 (72%)
30
31 (55%)
32; 33 (58% for each)
7 (99%); 8 (96%)

7 and 32 R = $(CH_2)_7CH{=}CH(CH_2)_7CH_3$; 8 and 33 R = $(CH_2)_7CH{=}CHCH_2CH{=}CH(CH_2)_4CH_3$

Scheme 4. Reagents: *a) NaN_3, n-Bu_4NHSO_4 cat., toluene; b) TMSBr, MeCN; c) $(COCl)_2$, DMF cat., DCM; d) H_3PO_3, pivaloyl chloride, pyridine; e) $CH_3(CH_2)_{15}I$, NaH, DMF/THF; f) CF_3COOH-water (9:1); g) TESCl, pyridine, DCM; h) oleoyl chloride for 32 (or linoleoyl chloride for 33), Et_3N, DMAP, pyridine; i) 3HF·Et_3N, MeCN/DCM; j) tri-imidazolyphosphine, MeCN/DCM; k) Et_3NHHCO_3, water (pH 7).*

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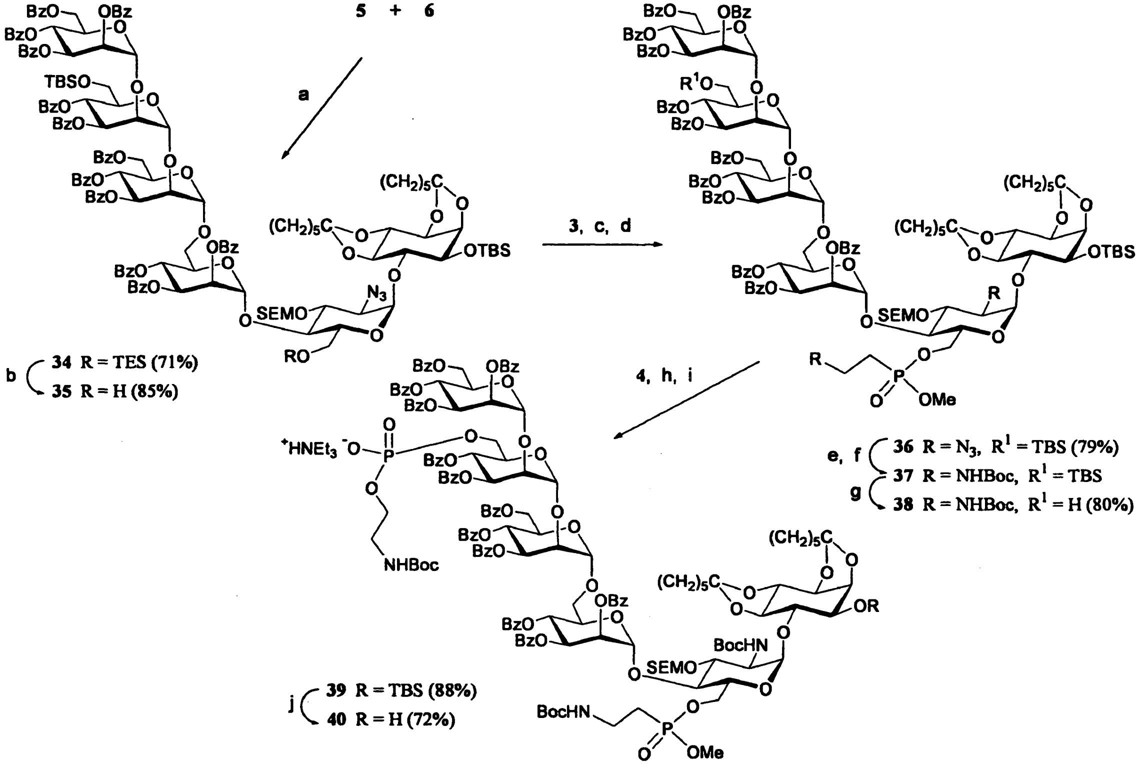
5 + 6
a
b
34 R = TES (71%)
35 R = H (85%)
3, c, d
e, f
36 R = N₃, R¹ = TBS (79%)
37 R = NHBoc, R¹ = TBS
g
38 R = NHBoc, R¹ = H (80%)
4, h, i
j
39 R = TBS (88%)
40 R = H (72%)

Scheme 5. Reagents: *a) $TMSOSO_2CF_3$, MS 4Å, DCM; b)* n-*Bu_4NF, AcOH, THF (20 °C, 1.5 h); c) 1*H-*tetrazole,* i-*Pr_2NEt, toluene; d) MeOH; e) Ph_3P, water, THF; f) Boc_2O, Et_3N, MeOH; g) $3HF{\cdot}Et_3N$, MeCN/THF; h) pivaloyl chloride, pyridine; i) I_2, pyridine-water; j)* n-*Bu_4NF, AcOH, THF (55 °C, 60 h).*

cleanly producing the 6""-OH glycan-inositol-phosphonate compound **38**[30] (80%; δ_P = 32.2, 32.8). Further, the introduction of the ethanolamine phosphate moiety was performed by the condensation of **38** with the hydrogenphosphonate derivative **4** (activated by pivaloyl chloride)[24] followed by oxidation (in situ) with iodine in aqueous pyridine. The phosphonate-phosphate block **39**[30] was isolated in 88% yield [δ_P = 0.4 (1 P), 31.9 (0.5 P), 32.1 (0.5 P)] prior the final desilylation with TBAF-AcOH (at 55 °C) gave the 1-OH glycoconjugate derivative **40**[30] [72%; δ_P = 0.3 (1 P), 31.9 (0.5 P), 32.3 (0.5 P)].

We reported earlier[25] that the presence in a molecule of phosphodiester units does not interfere with the next *O*-phosphorylation step by means of the H-phosphonate method (i.e., P-protection for phosphodiesters is not required). Indeed, compound **40** (containing a phosphodiester at O-6"") was successfully phospholipidated (Scheme 6) by pivaloyl chloride assisted reaction with the acylalkylglyceryl hydrogenphosphonate **7** followed by oxidation (in situ) with iodine to provide the fully protected oleic ester GPI **41**[30,31] [δ_P = –1.4 and –1.2 (0.5 P each), 0.0 and 0.2 (0.5 P each), 32.1 and 32.4 (0.5 P each)] in excellent yield. Similarly, the protected linoleic ester GPI **42**[30,31] [85%; δ_P = –1.3 and –1.2 (0.5 P each), 0.25 and 0.3 (0.5 P each), 32.1 and 32.4 (0.5 P each)] was made from **40** and the hydrogenphosphonate **8**. Both compounds **41** and **42** were immediately demethylated at the aminoethylphosphonate moiety with PhSH-Et_3N[26] to form GPI derivatives **43**[30,31] [76%; δ_P = –1.2, –0.1, 22.4] and **44**[31] [75%; δ_P = –2.1, –1.2, 22.7], respectively.

Global deprotection of compound **43** was performed in two steps. First, controlled *O*-debenzoylation with 0.05 M methanolic sodium methoxide (3 h) gave the partly protected GPI **45**, which was isolated in 40% yield by flash column chromatography (on SiO_2). The presence of the fatty ester in the molecule was clearly indicated by MALDI-TOF mass spectrometry data.[31] Subsequent cleavage of *O*-acetal and *N*-Boc protecting groups with 90% aq. TFA followed by purification by reverse phase chromatography (on Vydac C4-silica column in propan-1-ol/water/TFA, 10:90:0.05→95:5:0.05) provided the targeted oleic ester GPI **1** [90%; δ_P (D_2O) = –0.2, 0.4, 21.6]. The protected derivative **44** was converted to the linoleic ester GPI **2** [δ_P (D_2O) = –0.2, 0.4, 21.8] in a similar manner.

Synthetic strategy B

In our second approach (Scheme 7, retrosynthesis), a combination of various acetal type groups (dimethoxybutanediyl diacetal, cyclohexylidene and SEM), *N*-Boc carbamates and TBS ethers was applied as permanent protecting groups for glycan-inositol core assembly to ensure smooth final deprotection steps. An array of orthogonal blocking groups (required for further 'P-decoration') comprised TES ether for O-6 of D-glucosamine, phenoxyacetate for O-6 of D-mannose-3 and benzoic ester for O-1 of *myo*-inositol. The GPIs' glycan-inositol backbone was synthesised in a (3 + 3) manner from the mannotriose thioglycoside **47** and the pseudotrisaccharide block **48**. The P-containing synthons **3**, **4** and **7** (or **8**) were used (like in strategy A) for sequential introduction of the phosphonic and phosphoric esters.

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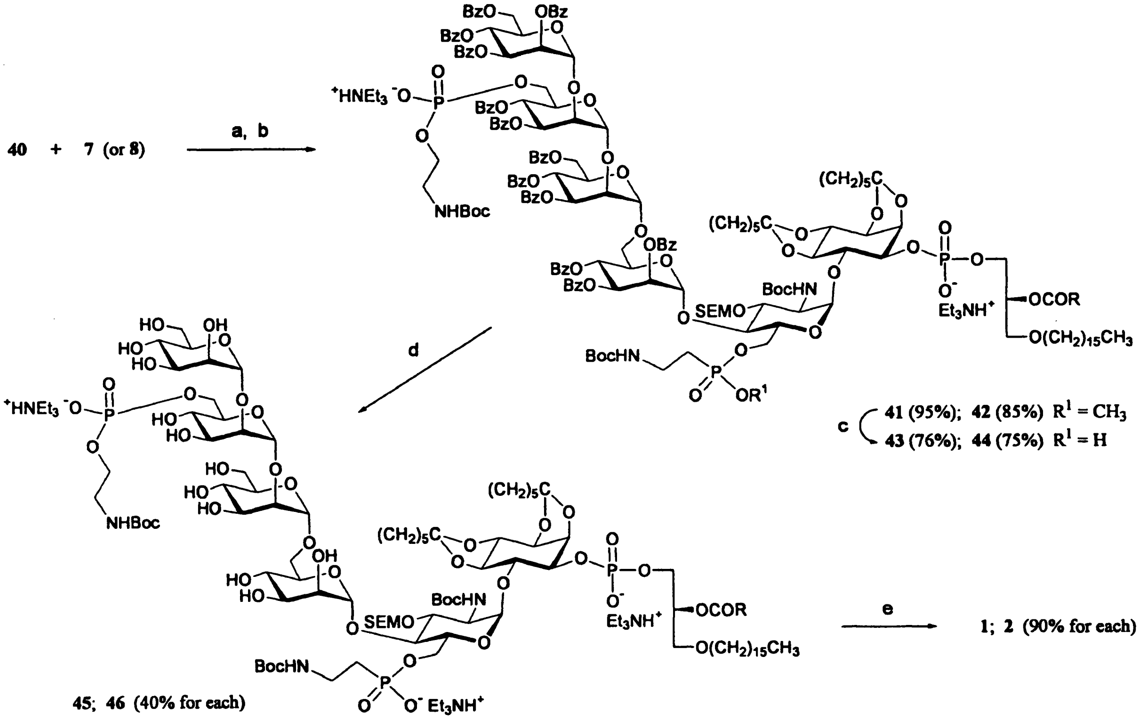
40 + 7 (or 8)
a, b
d
c
41 (95%); 42 (85%) R1 = CH3
43 (76%); 44 (75%) R1 = H
e
1; 2 (90% for each)
45; 46 (40% for each)
BzO
OBz
+HNEt3 -O
NHBoc
(CH2)5C
SEMO
BocHN
OR1
Et3NH+
OCOR
O(CH2)15CH3
HO
OH
O- Et3NH+

41, 43 and 45 R = $(CH_2)_7CH{=}CH(CH_2)_7CH_3$

42, 44 and 46 R = $(CH_2)_7CH{=}CHCH_2CH{=}CH(CH_2)_4CH_3$

Scheme 6. Reagents: *a) pivaloyl chloride, pyridine; b) I_2, pyridine-water; c) PhSH, Et_3N, DMF; d) 0.05 M NaOMe, MeOH; e) CF_3COOH-water (9:1).*

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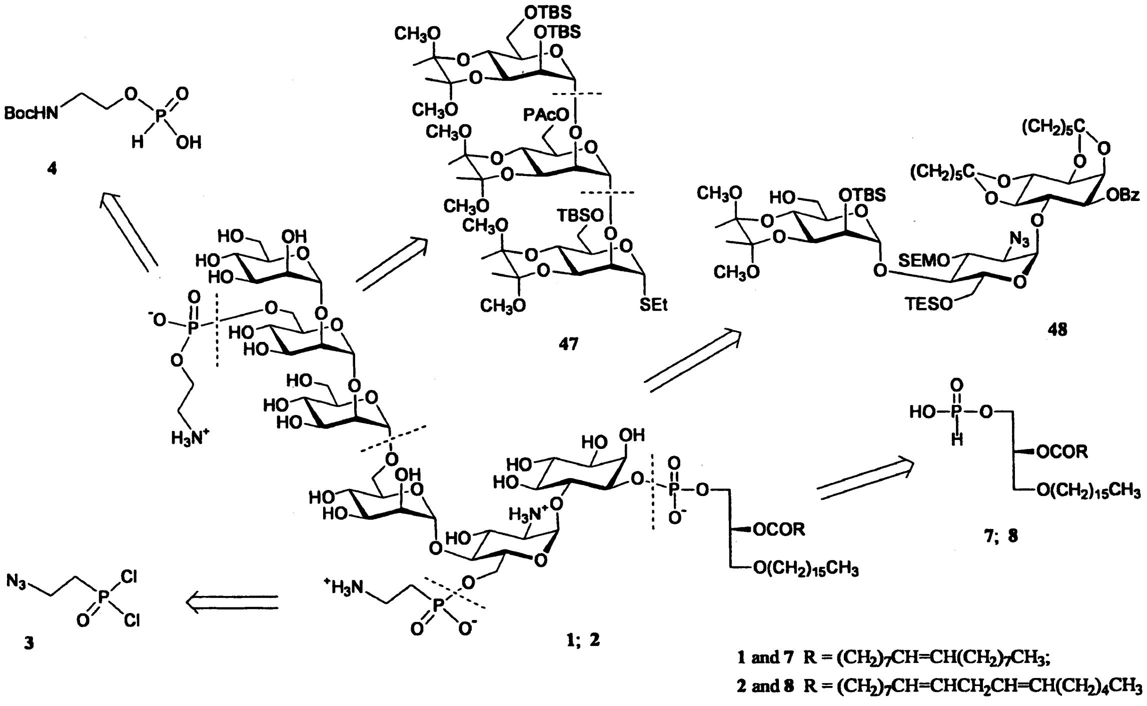

1 and 7 R = $(CH_2)_7CH{=}CH(CH_2)_7CH_3$;
2 and 8 R = $(CH_2)_7CH{=}CHCH_2CH{=}CH(CH_2)_4CH_3$

Scheme 7. Retrosynthetic scheme B. Pac = $PhOCH_2C(O)$.

In Frontiers in Modern Carbohydrate Chemistry; Demchenko, A.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2007.

The mannotriose block **47** was prepared from the monosaccharide derivatives **49-51**, which were assembled as shown on Scheme 8. Compounds **49** and **51**, in turn, were made from ethyl 3,4-*O*-(2',3'-dimethoxybutanediyl)-1-thio-α-D-mannoside **52** (in 90% yield each) using standard silylation methods. Compound **52** itself was made (90%) from ethyl 1-thio-α-D-mannoside using S. Ley's[27] method for diacetal formation, i.e., the reaction with butanedione and trimethyl orthoformate in MeOH in the presence of $BF_3{\cdot}Et_2O$. The preparation of the trichloroacetimidate **50** progressed also from **52** (in 75% overall yield) via consecutive benzoylation, anomeric deprotection (with Br_2 in DCM followed by Ag_2CO_3 assisted hydrolysis) and the reaction with CCl_3CN in the presence of 1,8-diazabicycloundec-7-ene (DBU). The disaccharide **53** (83%) was made by the coupling of the glycosyl acceptor **51** and the donor **50** in the presence of TMSOTf. It was then debenzoylated with MeONa in MeOH followed by selective monoacylation with phenoxyacetyl chloride (PAcCl) and *sym*-collidine at (–78 °C) to form the monohydroxyl derivative **54** (60%). Subsequent glycosylation with the thioglycoside **49** in the presence of methyl triflate (MeOTf) and 2,6-di-*tert*-butyl-4-methylpyridine (DBMP) gave the trisaccharide **47** in 65% yield. The result clearly reflected 'armed' nature of the glycosyl donor **49** compared to the 'disarmed' one for the thioglycoside **54** in the reaction.

The pseudotrisaccharide block **48** was synthesised (Scheme 9) using the thiomannoside donor **59** (prepared from **52** in two steps in 78% yield) and the azidoglucose-inositol derivative **58**. The latter was made starting from compound **23**: consecutive mild deacetylation, 4',6'-disilylation with tetraiso-propyldisiloxane dichloride and imidazole (→**55**, 68%) and the reaction with SEM-chloride and *i*-Pr_2NEt afforded the derivative **56** (92%). Following basic cleavage of the (–)-menthyl-carbonate and conventional benzoylation gave compound **57**, which was then successively desilylated (at O-4' and -6') with acetic acid buffered TBAF and silylated at O-6' with Et_3SiCl in pyridine-DCM to form the derivative **58** (48% overall yield from **23**). Condensation of compounds **59** and **58** in the presence of MeOTf and DBMP gave the pseudotrisaccharide **60** (83%), which was deprotected at O-6" with methylamine in ethanol-THF to provide the required synthon **48** (85%).

Glycosylation of the pseudotrisaccharide **48** with the mannotriose thioglycoside **47** (Scheme 10) was performed in the conditions similar to those used for the reaction **49** + **54** (but with longer reaction time: 4 days instead of 3 h) and provided the protected pseudohexasaccharide **61** in 75% yield(*). Subsequent cleavage of the TES group (with TBAF-acetic acid) smoothly gave the 6'-OH derivative **62** (75%), which was esterified with 2-azidoethylphosphonodichloridate **3** in the presence of 1*H*-tetrazole and *i*-Pr_2NEt followed by methanolysis to give the phosphonic diester **63**[30] (77%; a diastereomeric mixture; δ_P = 28.9, 29.6). It was then converted to the 6""-OH glycan-inositol-phosphonate derivative **65**[30] (72%; δ_P = 31.2, 32.5) by successive reduction of the azido groups with Ph_3P, *N*-protection with Boc-anhydride

---

(*) Coupling of compounds **47** and **48** in the presence of dimethylthiomethylsulfonium triflate and DBMP gave the same product **61** in 36% yield.

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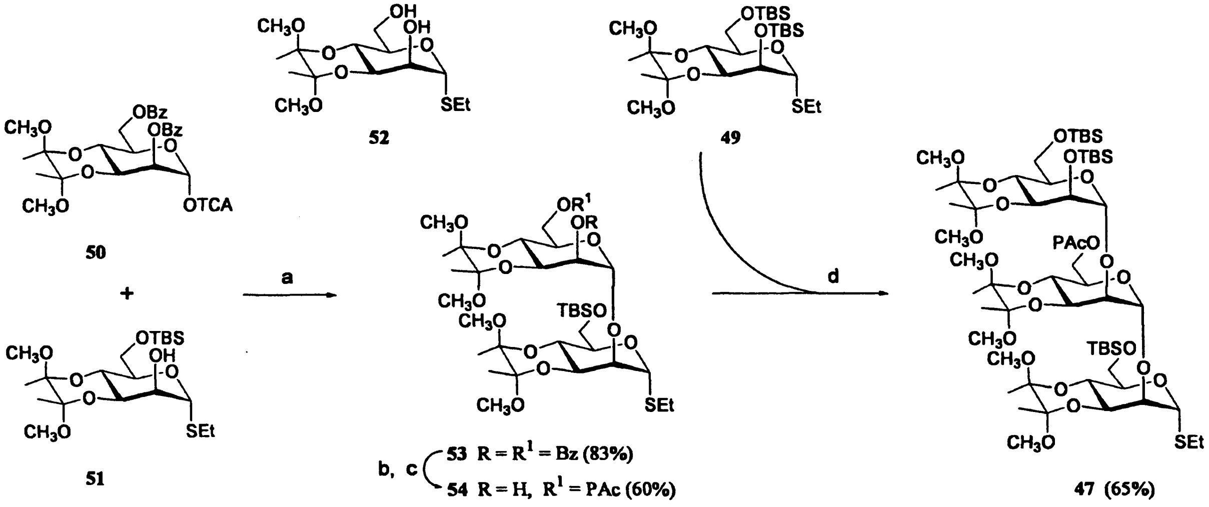

Scheme 8. Reagents: *a) TMSOSO$_2$CF$_3$, MS 4Å, Et$_2$O/DCM; b) 0.05 M NaOMe, MeOH; c) PAcCl,* sym-*collidine, DCM, (-78 °C); d) MeOSO$_2$CF$_3$, 2,6-di-*tert-*butyl-4-methylpyridine, MS 4Å, toluene (3 h).*

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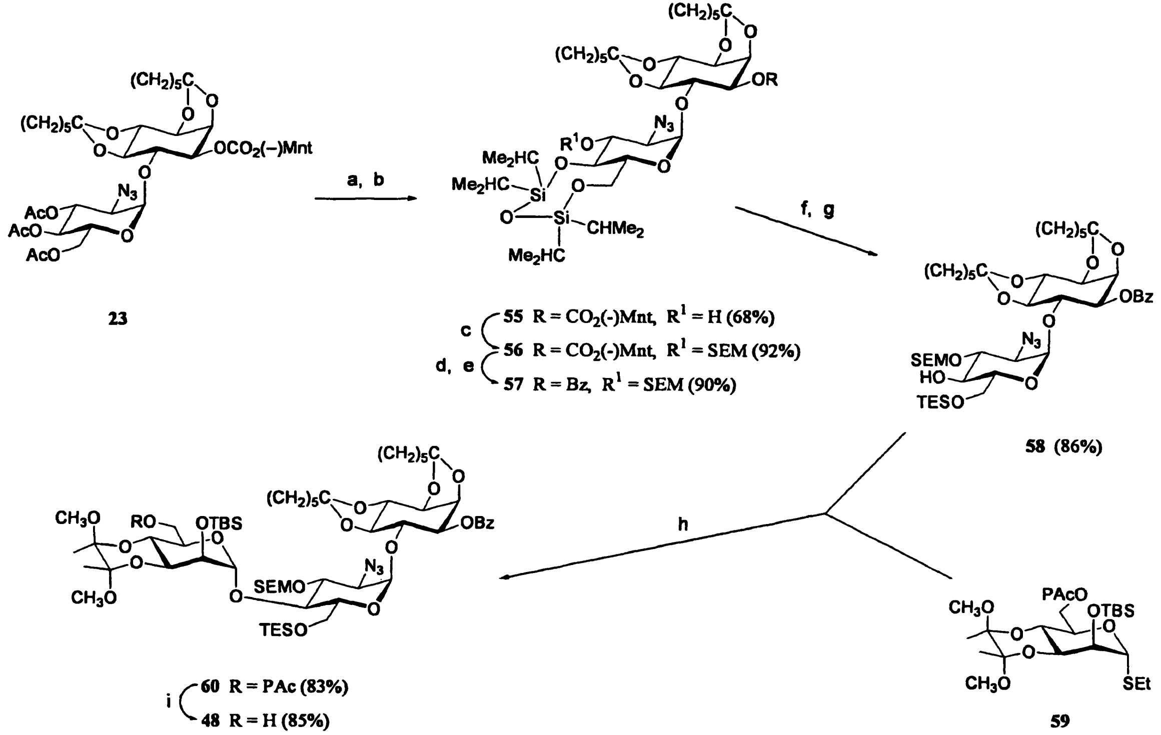
(CH2)5C
OCO2(−)Mnt
N3
AcO
AcO
AcO
23
a, b
Me2HC
Me2HC
R1O
OR
Si
CHMe2
Me2HC
c
d, e
55 R = CO2(-)Mnt, R1 = H (68%)
56 R = CO2(-)Mnt, R1 = SEM (92%)
57 R = Bz, R1 = SEM (90%)
f, g
OBz
SEMO
HO
TESO
58 (86%)
h
CH3O
PAcO
OTBS
SEt
59
RO
i
60 R = PAc (83%)
48 R = H (85%)

Scheme 9. Reagents: *a)0.02 M NaOMe, MeOH/DCM; b) (*i*-Pr$_2$ClSi)$_2$O, imidazole, MeCN; c) SEMCl,* i*-Pr$_2$NEt, DCM; d) 1 M NaOMe, MeOH/DCM; e) BzCl, pyridine; f)* n*-Bu$_4$NF, AcOH, THF; g) TESCl, pyridine, DCM, (-20 °C); h) MeOSO$_2$CF$_3$, 2,6-di-*tert*-butyl-4-methylpyridine, MS 4Å, toluene (7 days); i) MeNH$_2$, EtOH, THF.*

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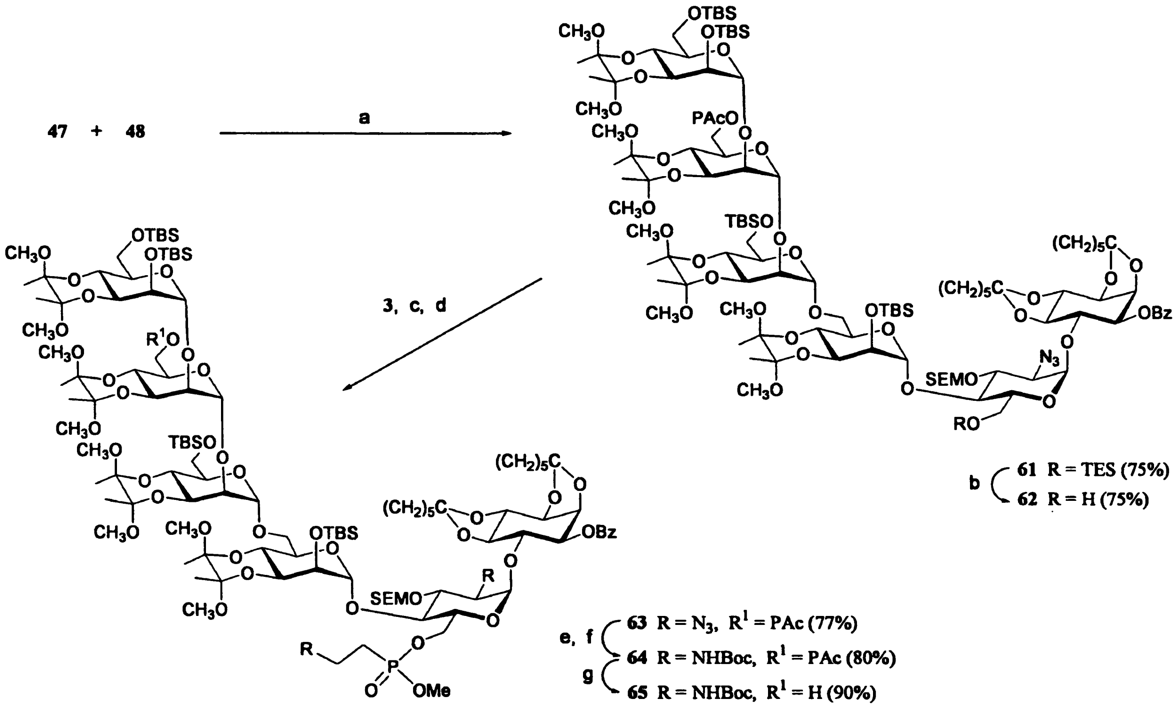
47 + 48
a
3, c, d
b
61 R = TES (75%)
62 R = H (75%)
e, f
g
63 R = N$_3$, R^1 = PAc (77%)
64 R = NHBoc, R^1 = PAc (80%)
65 R = NHBoc, R^1 = H (90%)
OTBS
CH$_3$O
PAcO
R^{1}O
TBSO
SEMO
N$_3$
RO
R
OBz
(CH$_2$)$_5$C
OMe

Scheme 10. Reagents: *a) MeOSO$_2$CF$_3$, 2,6-di-*tert*-butyl-4-methylpyridine, MS 4Å, toluene (4 days); b)* n-*Bu$_4$NF, AcOH, THF; c) 1*H-*tetrazole,* i-*Pr$_2$NEt, toluene; d) MeOH; e) Ph$_3$P, water, THF; f) Boc$_2$O, Et$_3$N, MeOH; g) MeNH$_2$, EtOH, THF.*

(→**64**)[30] and selective cleavage of the phenoxyacetate with methylamine in ethanol-THF.

The introduction of the ethanolamine phosphate moiety was performed by the reaction of **65** with the hydrogenphosphonate derivative **4** (Scheme 11) in the standard H-phosphonate condensation conditions described above for the preparation of compounds **39** and **41**. The phosphonate-phosphate block **66**[30] was isolated in 80% yield [δ_P = 1.0 (1 P), 30.8 (0.5 P), 32.1 (0.5 P)] prior the final orthogonal group cleavage (MeONa in MeOH) gave the 1-OH glycoconjugate derivative **67**[30] [95%; δ_P = 1.2 (1 P), 30.9 (0.5 P), 32.0 (0.5 P)]. Compound **67** was then phospholipidated by condensation with the acylalkylglyceryl hydrogenphosphonate **7** (in the same conditions as for the synthesis of compounds **39** and **41**) to provide the fully protected oleic ester GPI **68** [65%; δ_P = –1.5 and –1.2 (0.5 P each), 0.9 (1 P), 32.1 and 32.4 (0.5 P each)]. After demethylation at the aminoethylphosphonate moiety with PhSH-Et_3N,[26] it gave the GPI derivative **69**[31] [δ_P = –1.0, 0.8, 22.7]. For the global deprotection, compound **69** was, first, desilylated by treatment with $3HF{\cdot}Et_3N$ and pyridine in THF followed by cleavage of the acetal type and *N*-Boc protecting groups with 90% aq. TFA. Subsequent purification by reverse phase chromatography (as above in Syntheic strategy **A**) provided the targeted oleic ester GPI **1**.

The structures of the glycosylphospholipids **1** and **2** were supported by NMR spectroscopy and mass spectrometry data. The ^1H, ^{13}C as well as ^{31}P NMR spectra for compound **1** were almost identical to those for compound **2**. Full structural assignment of **2** (with exception for the acyl and alkyl residues) was performed by a combination of ^1H, ^{13}C, COSY, ROESY, TOCSY and HSQC spectroscopy and presented in Table 1. The ^{31}P NMR signals were assigned using ^1H,^{31}P HMQC technique. The molecular masses for the GPIs **1** and **2** were confirmed by MALDI-TOF and electrospray mass spectrometry.[30,31]

The synthetic GPIs **1** and **2** revealed biological activity: preliminary experiments using Toll-like receptor (TLR) transfected CHO cell lines showed they stimulated TLR2-transfected cells and not TLR4-transfected cells,[28] like naturally occurring tGPI.[29] A detailed biological evaluation of the compounds is currently in progress and will be published elsewhere in due course. To summarise, two novel synthetic strategies for the preparation of glycosylphosphotidylinositols, implicating the use of non-benzyl type protecting groups, were designed and showed their utility on the first syntheses of *T. cruzi* trypomastigote GPIs containing unsaturated fatty acids in the lipid moiety.

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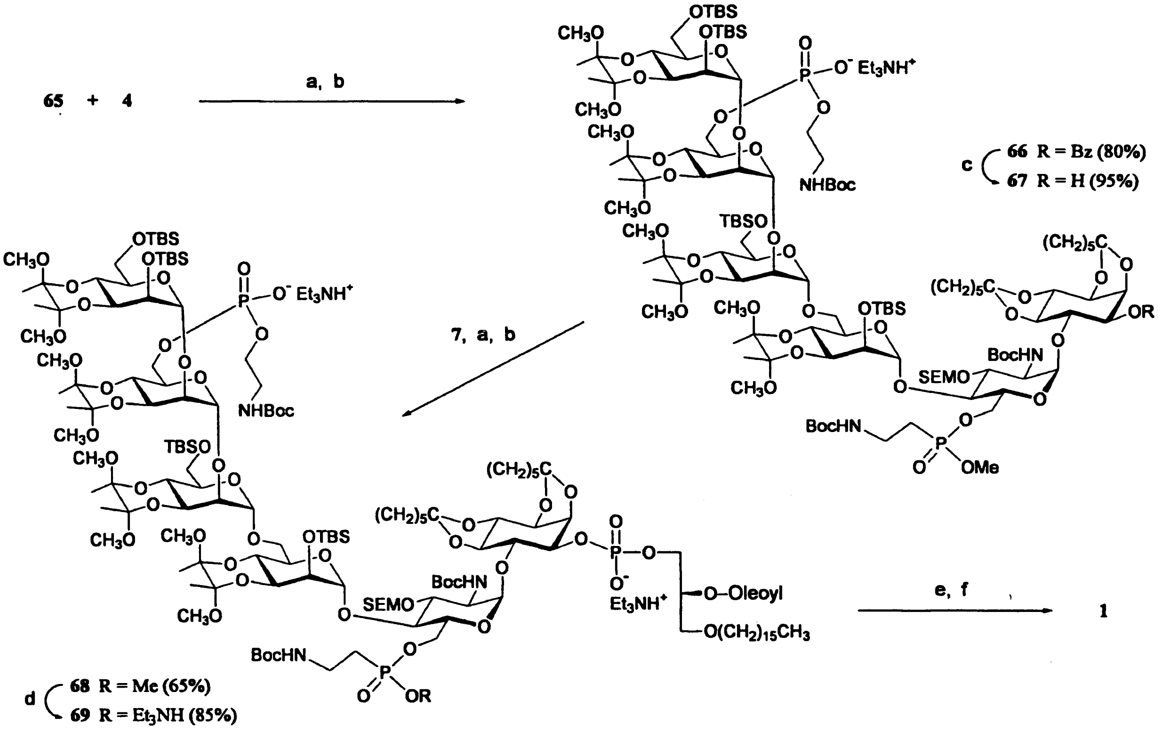
65 + 4
a, b
c
66 R = Bz (80%)
67 R = H (95%)
7, a, b
d
68 R = Me (65%)
69 R = Et3NH (85%)
e, f
1

Scheme 11. Reagents: *a) pivaloyl chloride, pyridine; b) I_2, pyridine-water; c) 0.25 M NaOMe, MeOH; d) PhSH, Et_3N, DMF; e) 3HF•Et_3N, pyridine, THF; f) CF_3COOH-water (9:1).*

Table 1. Correlation table of compound 2: ^{1}H, ^{13}C NMR and ^{31}P chemical shifts (in DMSO-D_6 at 30 °C; δ in ppm) at 500, 125 and 202 MHz, respectively.

Residue	H1	H2	H3	H4	H5	H6	P
	C1	C2	C3	C4	C5	C6	
Ino[a]	4.03	3.81	3.48	3.46	3.16	3.75	–0.6
	75.1	70.45	73.55	72.55	73.1	78.5	
$GlcNH_2$[b]	5.48	2.96	3.82	3.485	4.27	4.07 + 3.94	18.7
	94.3	53.8	70.1	76.25	69.2	63.1	
Man-15.105	3.86	3.48	3.60	3.19	3.85 + 3.62		
	101.25	71.8	70.65	66.1	70.8	65.6	
Man-24.85	3.78	3.70	3.45	3.62	3.67 + 3.42		
	98.2	77.05	70.1	67.1	72.4	61.2	
Man-3[c]	5.08	3.88	3.61	3.39	3.37	4.10 + 3.75	−1.5
	100.9	77.05	69.9	67.1	70.6	65.4	
Man-44.86	3.80	3.75	3.71	3.30	3.68 + 3.49		
	101.85	71.8	69.9	66.6	71.8	60.9	

[a] Additional signals of glycerol were present: CH_2-O-P, δ_H 3.80, δ_C 63.1; CH-O-acyl, δ_H 5.02, δ_C 71.0; CH_2-O-alkyl, δ_H 3.52 and 3.47, δ_C 68.9.

[b] Additional signals of the aminoethylphosphonate P-CH_2-CH_2-NH_3^+ were present: PCH_2, δ_H 2.01, δ_C 26.5; NCH_2, δ_H 3.06 and 3.00, δ_C 35.0.

[c] Additional signals of the ethanolamine phosphate P-O-CH_2-CH_2-NH_3^+ were present: $POCH_2$, δ_H 4.05 and 3.83, δ_C 65.7; NCH_2, δ_H 3.06 and 3.00, δ_C 40.0.

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30. MALDI-TOF-MS [positive mode, matrix: 2,5-dihydroxybenzoic acid] data for selected compounds: **36**, $C_{153}H_{173}N_6O_{45}PSi_3$ (2929.05): *m/z* 2951.71 $[M + Na]^+$, 2967.75 $[M + K]^+$; **37**, $C_{163}H_{193}N_2O_{49}PSi_3$ (3077.17): *m/z* 3100.19 $[M + Na]^+$, 3116.22 $[M + K]^+$; **38**, $C_{157}H_{179}N_2O_{49}PSi_2$ (2963.09): *m/z* 2986.15 $[M + Na]^+$, 3102.17 $[M + K]^+$; **39** (H^+-form), $C_{164}H_{193}N_3O_{54}P_2Si_2$ (3186.15): *m/z* 3209.38 $[M + Na]^+$, 3225.27 $[M + K]^+$, 3231.27 $[M - H + 2\ Na]^+$, 3247.35 $[M - H + Na + K]^+$; **40** (H^+-form), $C_{158}H_{179}N_3O_{54}P_2Si$ (3072.06): *m/z* 3095.30 $[M + Na]^+$, 3117.27 $[M - H + 2\ Na]^+$; **41** (H^+-form), $C_{195}H_{250}N_3O_{60}P_3Si$ (3714.56): *m/z* 3737.88 $[M + Na]^+$, 3759.86 $[M - H + 2\ Na]^+$; **2** (H^+-form), $C_{77}H_{144}N_3O_{41}P_3$ (1859.85): *m/z* 1923.00 $[M - H + 2\ Na + NH_3]^+$, 2018.60 $[M + Na + 2\ NH_3 + Et_3N]^+$.

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MALDI-TOF-MS [positive mode, matrix: α-cyano-4-dihydroxycinnamic acid] data for selected compounds: **63**, $C_{120}H_{203}N_6O_{44}PSi_5$ (2603.25): *m/z* 2628.34 $[M + Na]^+$, 2642.30 $[M + K]^+$; **64**, $C_{120}H_{223}N_2O_{48}PSi_5$ (2751.36): *m/z* 2774.87 $[M + Na]^+$, 2790.88 $[M + K]^+$.

ES-MS [positive mode] data for selected compounds: **42** (H^+-form), $C_{195}H_{248}N_3O_{60}P_3Si$ (3712.54): *m/z* 945.84 $[M + H + 3\ Na]^{4+}$; **43** (H^+-form), $C_{194}H_{248}N_3O_{60}P_3Si$ (3700.54): *m/z* 947.86 $[M + 4\ Na]^{4+}$; **65**, $C_{122}H_{217}N_2O_{46}PSi_5$ (2617.32): *m/z* 1329.88 $[M + H + Na + NH_3]^{2+}$, 2640.45 $[M + Na]^+$; **66** (H^+-form), $C_{129}H_{231}N_3O_{51}P_2Si_5$ (2840.38): *m/z* 2863.82 $[M + Na]^+$, 2885.97 $[M - H + 2\ Na]^+$; **67** (H^+-form), $C_{122}H_{227}N_3O_{50}P_2Si_5$ (2736.36): *m/z* 2759.30 $[M + Na]^+$, 2838.60 $[M + H + Et_3N]^+$; **2** (H^+-form), $C_{77}H_{144}N_3O_{41}P_3$ (1859.85): *m/z* 1240.50 $[2\ M + 3\ H]^{3+}$, 1791.90 $[M - NH_2C_2H_4PO_2H + H + Na + NH_3]^+$, 1808.84 $[M - NH_2C_2H_4PO_2H + H + K + NH_3]^+$, 1847.70 $[M - NH_2C_2H_4PO_2H + Na + K + 2\ NH_3]^+$.

ES-TOF-MS [positive mode] data for compound **1** (H^+-form), $C_{77}H_{146}N_3O_{41}P_3$ (1861.86): *m/z* 931.94 $[M + 2\ H]^{2+}$, 942.92 $[M + H + Na]^{2+}$.

31. ES-MS [negative mode] data for selected compounds: **41** (H^+-form), $C_{195}H_{250}N_3O_{60}P_3Si$ (3714.56): *m/z* 3713.36 $[M - H]^-$, 3735.32 $[M - 2\ H + Na]^-$; **42** (H^+-form), $C_{195}H_{248}N_3O_{60}P_3Si$ (3712.54): *m/z* 3711.24 $[M - H]^-$; **43** (H^+-form), $C_{194}H_{248}N_3O_{60}P_3Si$ (3700.54): *m/z* 3699.29 $[M - H]^-$, 3721.17 $[M - 2\ H + Na]^-$; **44** (H^+-form), $C_{194}H_{246}N_3O_{60}P_3Si$ (3698.53): *m/z* 3697.13 $[M - H]^-$, 3719.09 $[M - 2\ H + Na]^-$; **69** (H^+-form), $C_{158}H_{296}N_3O_{56}P_3Si_5$ (3364.84): *m/z* 1128.40 $[M - 4\ H + Na]^{3-}$, 1692.60 $[M - 3\ H + Na]^{2-}$.

MALDI-TOF-MS [negative mode, matrix: 2,5-dihydroxybenzoic acid] data for selected compounds: **45** (H^+-form), $C_{110}H_{200}N_3O_{48}P_3Si$ (2452.23): *m/z* 2451.45 $[M - H]^-$, 2473.42 $[M - 2\ H + Na]^-$, 2495.40 $[M - 3\ H + 2\ Na]^-$; **1** (H^+-form), $C_{77}H_{146}N_3O_{41}P_3$ (1861.86): *m/z* 1861.09 $[M - H]^-$, 1883.11 $[M - 2\ H + Na]^-$, 1899.03 $[M - 2\ H + K]^-$, 1905.15 $[M - 3\ H + 2\ Na]^-$.

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Chapter 17

Glycodiversification for the Synthesis of Neomycin and Kanamycin Class Aminoglycoside Antibiotics

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Aminoglycosides are an important resource against infectious diseases. However, the prevalence of aminoglycoside resistant bacteria has limited their use and resulted in the need for novel aminoglycosides. Our group has synthesized two libraries of aminoglycosides: pyranmycin and kanamycin analogs, utilizing the glycodiversification concept. Several of them show promising activity against both susceptible and resistant strains of *E. coli*. The structure activity relationship of these compounds further provides information for the future designs of aminoglycoside.

Introduction

The emergence of drug resistant microorganisms represents stringent public health problems. (*1-5*) The annual cost of treating antibiotic resistant infections in the United States alone has been estimated to be as high as $30 billion. (*6*, *7*) In responding to the call for new drug development, aminoglycosides, which have the advantage of high and broad-spectrum activity, are one of the ideal solutions against infectious diseases.

Aminoglycosides are a group of structurally diverse antibiotics consisting of various numbers of normal and unusual sugars. Neomycin and kanamycin are two of the most studied aminoglycoside antibiotics (Figure 1). Neomycin belongs to a group of aminoglycosides containing a 4,5-disubstituted 2-deoxystreptamine core (ring II), while kanamycin contains a 4,6-disubstituted 2-deoxystreptamine core. (*8*, *9*) Neamine (rings I and II) is the common core for both neomycin and kanamycin class antibiotics. These two types of antibiotics exert their antibacterial activity by binding selectively toward the A-site decoding region of 16S rRNA, and thereby disrupting the protein synthesis.

Extensive effort documented in the literature has been devoted into the synthesis of new aminoglycosides with the goal of providing novel therapeutic options against infectious diseases. In general, there are two types of strategies reported for the syntheses of new aminoglycoside antibiotics. The first one is modification of existing aminoglycosides, which is well-documented in the book chapter by Mobashery and co-workers. (*10*) The second approach is to apply the glycosylation strategies on selected cores, such as 2-deoxystreptamine (ring II) and neamine (rings I and II), and create libraries of new aminoglycosides. This strategy of "swapping" the originally attached carbohydrate components with the synthetic carbohydrates, followed by rationale-based modifications, is termed glycodiversification or glyco-optimization. (*11*, *12*)

The primary obstacle of utilizing the concept of glycodiversification for the library synthesis of novel aminoglycosides is the synthesis of structurally diverse unusual sugar donors needed for stereoselective glycosylation. We have employed divergent synthesis for the construction of two libraries of unusual sugar donors: the phenylthioglycosyl donors and the glycosyl trichloroacetimidates for the construction of kanamycin and pyranmycin (neomycin class), respectively. The former library of glycosyl donors will favor the formation of essential α-glycosidic bond in kanamycin upon glycosylation while the latter will favor the pivotal β-glycosidic bond in pyranmycin.

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Library Synthesis of Pyranmycin

Design of Pyranmycin

Pyranmycin is a group of synthetic aminoglycoside antibiotics containing 4,5-disubstituted 2-deoxystreptamine core, which is the character of neomycin class antibiotics. (*12-16*) However, it differs from neomycin by containing a pyranose in place of furanose at the O-5 position of neamine. Since the glycosidic bond of a furanose (five-membered ring) is more acid labile than that of a pyranose (six-membered ring), (*17*, *18*) therefore, it is conceivable that the replacement of the furanose ring (III) with an aminopyranose (Figure 2) will increase the much-needed acid-stability and, hopefully, reduce the cytotoxicity due to the lower administered dosage needed for achieving the therapeutically effective concentration of antibiotics.

First Generation of Pyranmycin

Pyranmycin which contains various D-pyranoses and L-pyranoses on ring III have been synthesized (Scheme 1). (*13*, *14*) The β-glycosidic bond between

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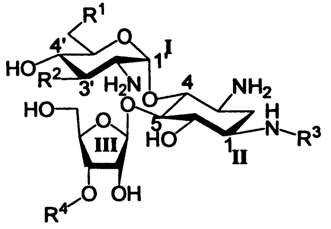
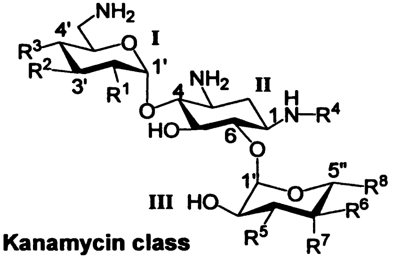


Neomycin class

	R^1	R^2	R^3	R^4
Neomycin B	NH_2	OH	H	DAG
Paromomycin I	OH	OH	H	DAG
Lividomycin B	OH	H	H	DAG
Butirosin B	NH_2	OH	AHB	H
Ribostamycin	NH_2	OH	H	H

DAG: 2,6-diamino-2,6-dideoxy-α-L-glucopyranosyl



Kanamycin class

	R^1	R^2	R^3	R^4	R^5	R^6	R^7	R^8
Kamanycin A	OH	OH	OH	H	NH_2	OH	H	CH_2OH
Gemtamicin A	NH_2	OH	OH	H	$NHCH_3$	H	OH	H
Tobramycin	NH_2	H	OH	H	NH_2	OH	H	CH_2OH
Amikacin	OH	OH	OH	AHB	NH_2	OH	H	CH_2OH
Arbekacin	NH_2	H	H	AHB	NH_2	OH	H	CH_2OH

AHB: (S)-4-amino-2-hydroxybutyryl

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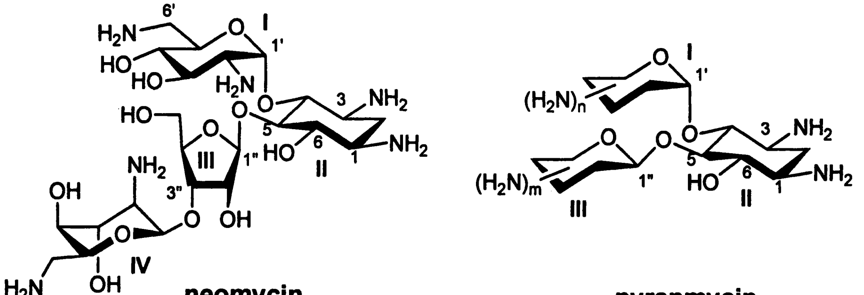
Figure 1. Structures of Neomycin and Kanamycin Classes Aminoglycosides



neomycin (ring III with **furanose**)

pyranmycin (ring III with **pyranose**)

Figure 2. Structural Relationship between Neomycin and Pyranmycin

rings II and III was directed by neighboring group of glycosyl trichloroacetimidate donors. The antibacterial assay result from the first generation of pyranmycin reveals three leads, **TC005**, **TC006**, and **TC010**. The presence of 6"-CH_3 was found to be an important factor in increasing the antibacterial activity for D-pyranose while 6"-CH_2OH is important for L-pyranose.

Modification Using Branched-chain Pyranose

The results from the studies of structure-activity relationship (SAR) of first generation pyranmycin show that the presence of 6-deoxy-D-glucopyranose as ring III of pyranmycin is essential for antibacterial activity, while the presence of a 6-amino-6-deoxy-L-idopyranose as ring III also manifest significant activity. Thus, we are interested in a design that combines both structural features, hypothesizing that such a structure should lead to an improved antibacterial activity (Figure 3).

The attempts of making the identical desired compound in Figure 3 were unsuccessful. Nevertheless, compound **TC054**, which contains hydroxyl group at C-7" position, was prepared successfully instead (Figure 4). (*16*) The combined structural features from **TC020** and **TC036**, **TC054** can still be used to examine our hypothesis.

The synthesized new members were assayed against *Escherichia coli* (ATCC 25922), and *Staphylococcus aureus* (ATCC 25923) using neomycin B as the control to generate the minimum inhibitory concentration (MIC) (Table I). (*19*) However, to our surprise, **TC054** manifested lower activity. The molecular modeling structures did not show significant structural perturbation due to the introduction of the 5-*C* methyl group on **TC054**. The binding score of **TC054** also cannot explain the unexpected decreased in antibacterial activity (Table 1).

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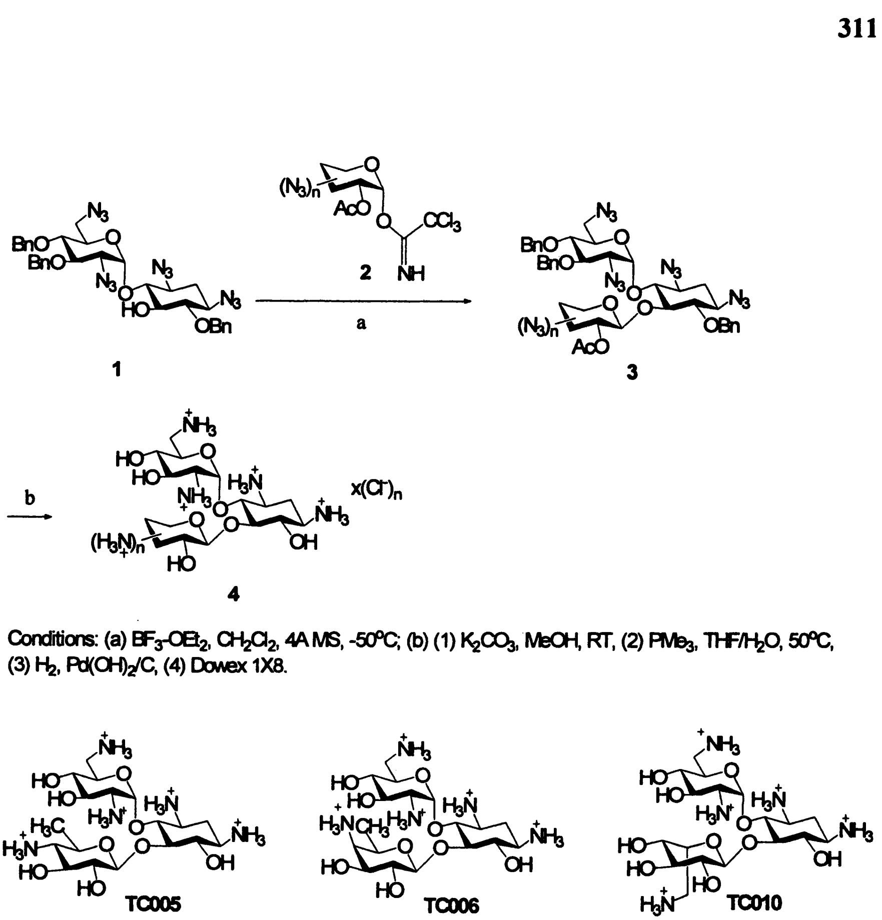

Conditions: (a) BF_3-OEt_2, CH_2Cl_2, 4A MS, -50°C; (b) (1) K_2CO_3, MeOH, RT, (2) PMe_3, THF/H_2O, 50°C, (3) H_2, $Pd(OH)_2/C$, (4) Dowex 1X8.

Scheme 1. Synthesis of Designed Pyranmycin

Table I. Minimum Inhibitory Concentration and Binding Scores of the Synthesized Pyranmycins[a]

Compounds	*E.coli*	*S. aureus*	Binding Score[b]
Neomycin B	2	0.3	-474.30
TC054	87	27	-317.60
TC020	19	13	-314.50
TC036	11	3	-320.00

[a] Unit: μM

[b] The tendency in binding: the lower the number, the better the binding

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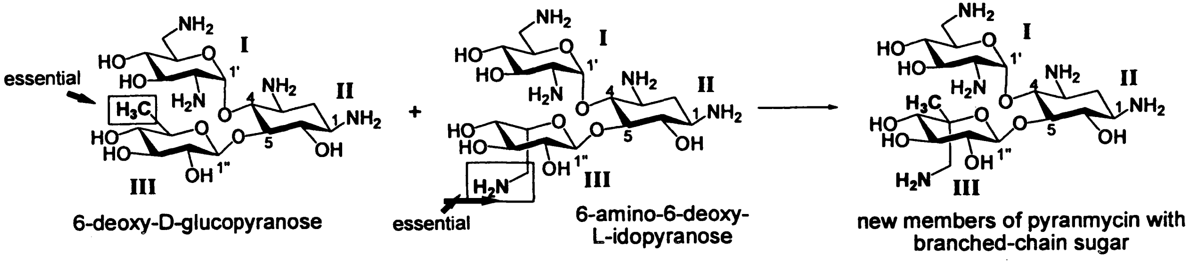
essential
I
II
III
1'
1"
4
5
1
+
essential
6-deoxy-D-glucopyranose
6-amino-6-deoxy-L-idopyranose
new members of pyranmycin with branched-chain sugar

Figure 3.

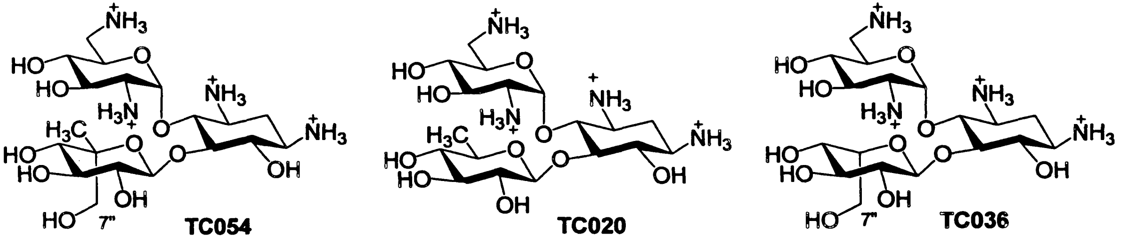
7"
TC054
TC020
TC036

Figure 4.

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Thus, these results may imply that other factors, for example, the mechanistic details of aminoglycoside uptake, permeability of aminoglycoside, and solvation, could have significant effect on the activity of aminoglycosides, and deserve more investigation.

3',4'-Dideoxygenation

APH(3'), which catalyzes phosphorylation at the 3'-OH of both neomycin and kanamycin classes of aminoglycosides, is one of the most prevalent aminoglycoside modifying enzymes. It renders the phosphorylated adduct incapable of binding toward the ribosomal target. Dideoxygenation at 3' and 4' position has been reported to be an effective modification against the action of APH(3'). (*20*, *21*) The design has led to the syntheses and discovery of tobramycin, (*22*, *23*) arbekacin, (*24*) and other similar aminoglycosides (Figure 1). (*25*)

The reported synthetic methods for 3',4'-dideoxygenation are, however, not compatible with the presence of azido groups. Thus, we have developed a new method for the synthesis of desired 3',4'-dideoxy-1,3,2',6'-tetraazidoneamine (Scheme 2). (*26*) The dideoxygenation was carried out at very wild condition via triflation of the 3', 4'-diol, followed by treatment of NaI and $Na_2S_2O_3$,.

Regioselective protection of C-6 hydroxyl group of compound **8** furnished compound **10**, which was glycosylated with the corresponding trichloroacetimidate donor, **2a**, generating **11**. Staudinger reaction and hydrogenation leads to the synthesis of 3',4'-dideoxypyranmycin adduct **RR501**. A library of aminoglycoside bearing 3',4'-dideoxygenation can be prepared in similar fashion.

RR501 was assayed against aminoglycoside susceptible and resistant strains of *Escherichia coli* using ribostamycin, and butirosin as the controls (Table II). As expected, **RR501** is more active against resistant strains than ribostamycin, which has a 3'-OH group. However, it is less active than butirosin which contains (S)-4-amino-2-hydroxybutyryl (AHB) group at N-1. Information from the MIC values has prompted the effort into the synthesis of pyranmycin with AHB group at N-1 and 3',4'-dideoxygenation (or 3'-deoxygenation).

N-1 Modification

Attaching functionalities at the N-1 position of the 2-deoxystreptamine on kanamycin or neomycin class antibiotics has been shown to be one of the most effective methods against aminoglycoside resistant bacteria. (*27-29*) This strategy has led to the development of semi-synthetic amikacin that has a AHB group at N-1 position (Figure 1). The synthesis of kanamycin and neomycin

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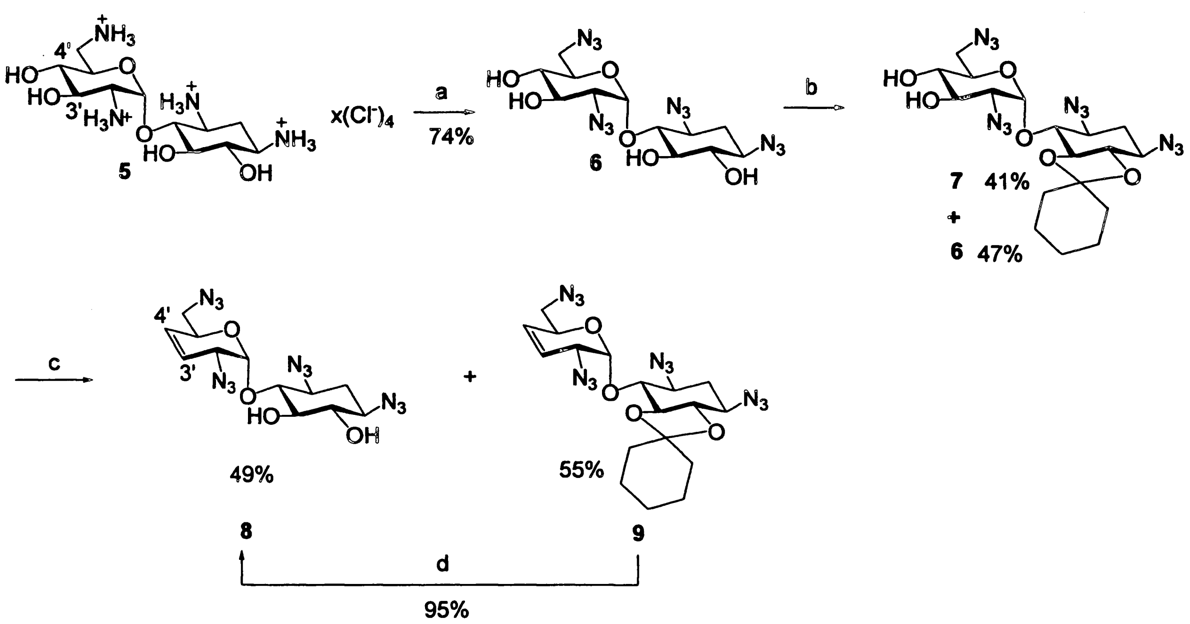

Conditions: (a) TfN_3, $CuSO_4$, H_2O, CH_2Cl_2, (b) Cyclohexone dimethyl ketal, $TsOH\text{-}H_2O$, CH_3CN, (c) (1) Tf_2O, pyr., CH_2Cl_2; (2) $Na_2S_2O_3$, NaI, acetone, (d) H_2O, HOAc, dioxane, 65°C.

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Scheme 2. Synthesis of Neamine Acceptor

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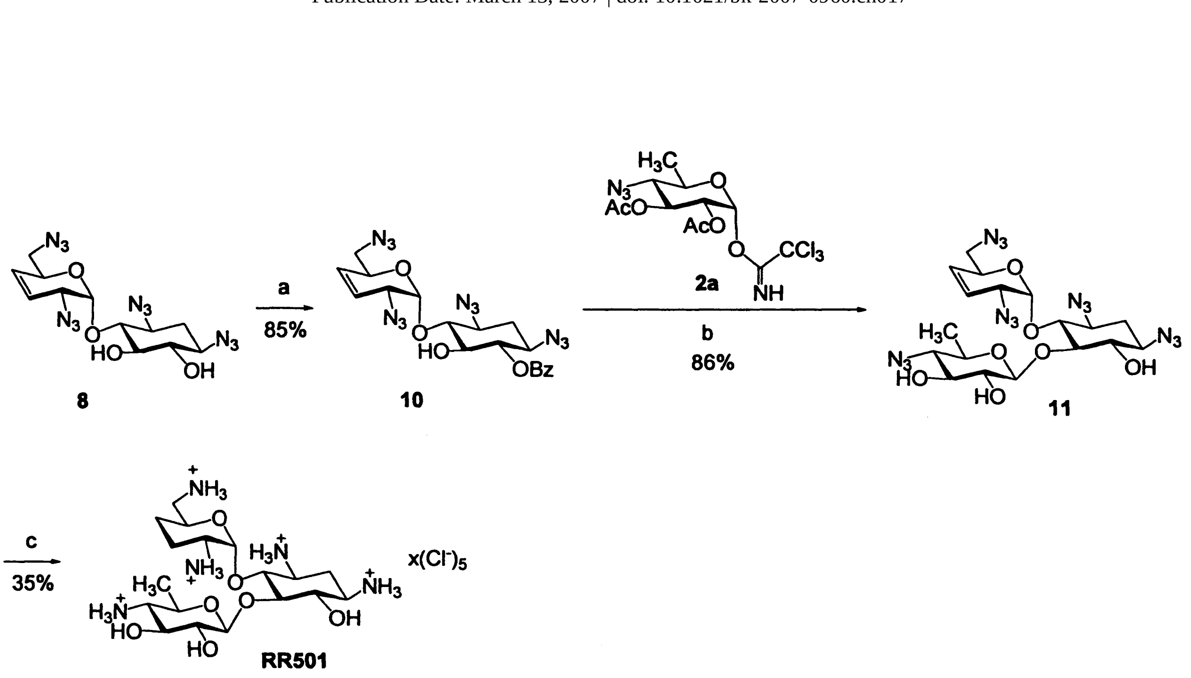

Conditions: (a) BzCl, CH_2Cl_2, -50°C; (b) (1) BF_3-OEt_2, CH_2Cl_2, 4A MS, (2) NaOMe, MeOH; (c) (1) PMe_3, THF, (2) H_2, $Pd(OH)_2/C$, $HOAc/H_2O$, (3) Dowex 1X8-200(Cl^-).

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Scheme 3. Synthesis of 3',4'-Dideoxy Pyranmycin

Table II. Minimum Inhibitory Concentration of the Synthesized Aminoglycoside[a]

Compounds	Strains		
	E. coli (TG1)	*E. coli* (TG1) (pSF815)[b]	*E. coli* (TG1) (pTZ19U-3)[c]
Ribostamycin	2	16	Inactive
Butirosin	0.5	0.5	0.5
RR501	8	4	4

[a]: Unit: μg/mL; [b]: plasmid encoded for AAC6'/APH2"; [c]: plasmid encoded for APH(3')-I

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class aminoglycosides with N-1 modification can be achieved via chemical (*27-30*) or enzymatic (*31*) methods. However, no applicable chemical methods can be used to regioselectively reduce the N-1 azido group of 1,3,2',4'-tetraazidoneamine, which is essential for introducing the modification at N-1 position of kanamycin and pyranmycin class aminoglycosides.

It has been reported that the electron-withdrawing protecting groups will enhance the reactivity of their vicinal azido group toward the Staudinger reaction. Thus, we began to examine the possibility of introducing electron-withdrawing protecting groups, such as acyl groups, at *O*-5 and/or *O*-6 positions for selective reduction of the N-1 azide group. (*32*) After screening a panel of acyl groups, the synthesis of important intermediate 3',4'-Di-*O*-benzyl-1-*N*-*tert*-butoxycarbonyl-3,2',6'-triazidoneamine was accomplished (Scheme 4).

Selective benzoylation of compound **15** at *O*-6 directs the glycosylation at *O*-5 (Scheme 5). Deprotection of Boc, followed by coupling with the Cbz-protected AHB side chain and glycosylation, yielded compound **18**. Staudinger reduction of the remaining azido groups followed by hydrogenation provided the final product **JT005**.

The constructed N-1 modified pyranmycin was assayed against aminoglycoside susceptible and resistant strains of *Escherichia coli* using ribostamycin, and butirosin as the controls. From the MIC values, the synthesized aminoglycoside with the attachment of AHB group at N-1 regain the activity against both resistant strains of bacteria (Table III).

Library Synthesis of Kanamycin B Analogs

Design of Kanamycin Analogs

Kanamycin, like neomycin, exerts prominent antibacterial activity against both gram positive and gram negative susceptible strains of bacteria. However, the clinical effectiveness of kanamycin has become obsolete due to the prevalence of aminoglycoside resistant bacteria. (*33*, *34*) As one of the antidotes

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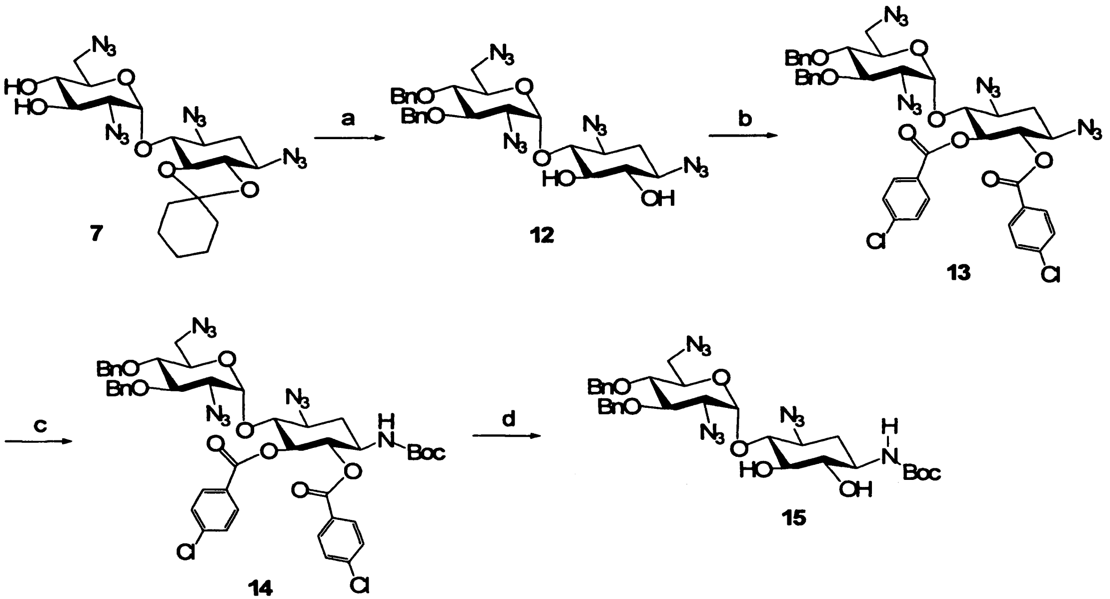

Conditions: (a) (1) BnBr, NaH, TBAI, THF, (2) dioxane, H_2O, HOAc; (b) 4-ClBzCl, Et_3N, CH_2Cl_2, DMAP, reflux; (c) 1.0 M PMe_3 in tolueme (1.1 eq.), Boc-ON (2.4 eq.), toluene (anhydrous), -78°C - 10°C; (d) NaOMe in MeOH (0.5M), MeOH/THF (5/1);

Scheme 4.

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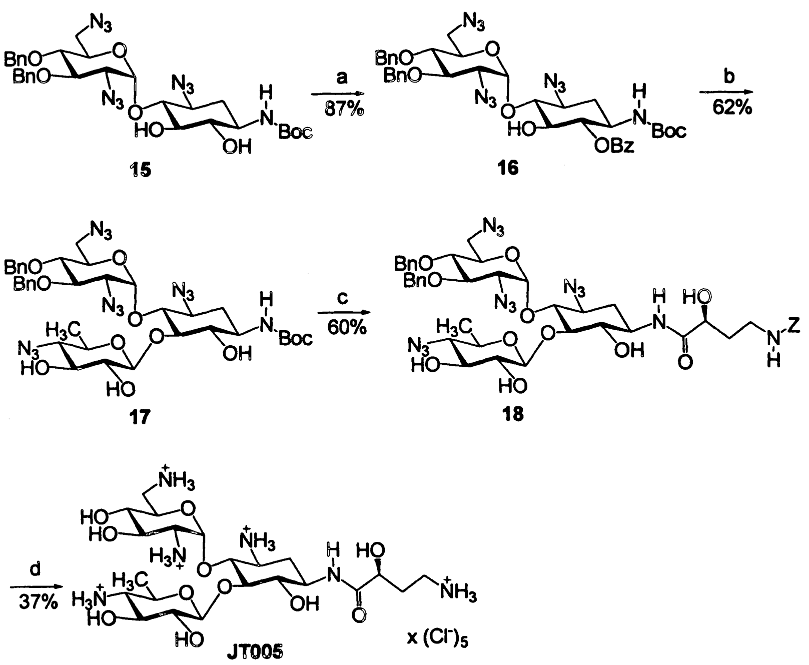

Conditions: (a) BzCl, CH_2Cl_2, -50°C; (b) (1) glycosyl donor **2a**, BF_3-Et_2O, CH_2Cl_2, (2) NaOMe, THF, MeOH; (c) (1) TFA, CH_2Cl_2, (2) *N*-Cbz AHB, DCC, *N*-hydroxylsuccinamide, THF, Et_3N; (d) (1)PMe_3, THF, H_2O, (2) H_2, $Pd(OH)_2/C$, $HOAc/H_2O$ (1/4), (3) Dowex 1X-8 (Cl^- form).

Scheme 5.

Table III. Minimum Inhibitory Concentrations of the Synthesized Aminoglycoside[a]

Compounds	Strains		
	E. coli (TG1)	*E. coli* (TG1) (pSF815)[c]	*E. coli* (TG1) (pTZ19U-3)[d]
Butirosin	1	0.25	0.5
Ribostamycin	2	8	Inactive
JT005	4	4	4
TC005[b]	8	8	Inactive

[a]: Unit: μg/mL
[b]: The corresponding pyranmycin without AHB group at N-1
[c]: plasmid encoded for AAC6'/APH2"
[d]: plasmid encoded for APH(3')-I

against the problem of drug resistance, numerous chemical modifications of kanamycin have been reported with the goal of reviving its activity toward resistant bacteria. (*21*, *35-37*) Except for a few publications, (*38*, *39*) the majority of derivatives have been directed into the modification on kanamycin scaffold, which limits the options for structural modifications.

First Generation of Kanamycin B Analogs

Similar to the construction of pyranmycin, a library synthesis of novel kanamycin B analogues has been developed. (*40*) The neamine acceptor, **22**, undergoes regiospecific glycosylation at O-6 position, resulting in the desired 4,6-disubstituted 2-deoxystreptamine motif (Scheme 6). (*39*, *41*, *42*) The glycosylation donors have benzyl or azido groups at the C-2 position, which favor the formation of an α-glycosidic bond under the influence of anomeric and solvent effects. (*43*) The kanamycin analogues were tested against *E. coli* (ATCC 25922), and *S. aureus* (ATCC 25923) using kanamycin B as the control, revealing a lead **JL027**.

Modification at O-4" Position

Combined SAR information from first generation of kanamycin B analogs allows us to identify 4"-OH of ring III as the optimal site for further modification. Since kanamycin exerts its antibacterial activity by binding toward rRNA, a highly negatively-charged molecule due to the phosphate diester backbone, it is our expectation that by introducing more positively-charged side chain at the O-4" position, an increase in the antibacterial activity can be obtained.

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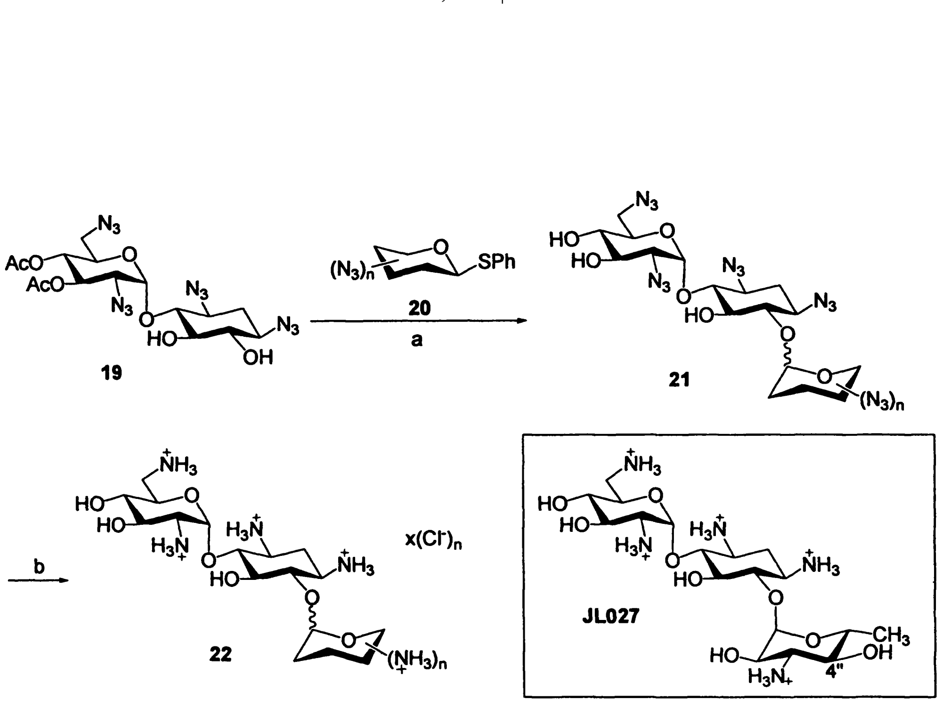
AcO
AcO
N_3
N_3
O
HO
N_3
N_3
OH
19
$(N_3)_n$
O
SPh
20
a
HO
HO
N_3
N_3
N_3
N_3
HO
O
21
$(N_3)_n$
b
$\overset{+}{N}H_3$
HO
HO
$H_3\overset{}{N}$
$H_3\overset{+}{N}$
$\overset{+}{N}H_3$
HO
O
22
$(\overset{+}{N}H_3)_n$
$x(Cl^-)_n$
$\overset{+}{N}H_3$
HO
HO
H_3N
$H_3\overset{+}{N}$
$\overset{+}{N}H_3$
HO
O
JL027
CH_3
OH
HO
H_3N_+
4"

Conditions: (a) (1) NIS, TfOH, $Et_2O:CH_2Cl_2$ (3:1), -70°, (2) NaOMe, MeOH, THF; (b) (1) PMe_3, THF/H_2O, (2) H_2, $Pd(OH)_2/C$, $HOAc/H_2O$, (3) Dowex 1X8-200(Cl^- form).

Scheme 6.

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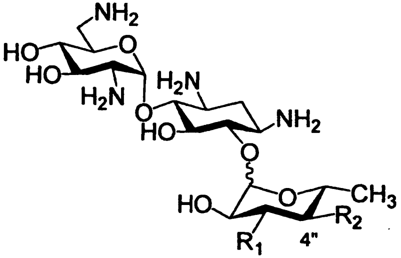


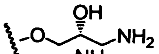
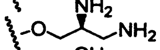
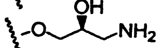
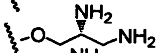
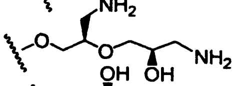
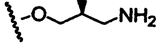
Compound	R_1	R_2	α : β
JL028	OH	$-O(CH_2)_3NH_2$	30: 1
JL029	OH		only α
JL030	OH		20:1
JL031	OH		only α
JL032	OH		only α
JL033	OH		35: 1
JL034	NH_2		25: 1

Figure 5. Structures of Kanamycin Analogs with Extended Arm

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Initial studies (**JL028** – **JL032**) were accomplished using 6-deoxyglucopyranose because of its easiness in preparation. After the identification of the optimal structural component at O-4”, the desired 6-deoxy-3-aminoglucopyranose was employed (**JL034**). Synthesis of **JL033** was designed to test whether an enlarged side chain could render the kanamycin construct a poor substrate for the aminoglycoside-modifying enzymes, and, thereby, regain the activity against resistant bacteria (Figure 5).

These O-4” modified kanamycin B analogs were assayed as described previously. From the results of antibacterial assay, we noticed that there is no significant difference in the activity from analogs with the variation of functional groups (OH or NH_2) and stereocenter of side-chain at O-4”, albeit an increase in activity has been observed. No activity was obtained when these analogs were tested against aminoglycoside-resistant bacteria. The lack of activity of **JL033** against aminoglycoside resistant bacteria suggests that the attachment of large but flexible side chain at O-4” position is, perhaps, an ineffective design against resistant bacteria strains.

3’,4’-Dideoxygenation

Synthesis of 3’,4’-dideoxygenated kanamycin analogs starts from 3’,4’-dideoxy-1,3,2’,6’-tetraazidoneamine. The glycosyl donor, **20a**, was selected based on the ease of synthesis and the higher antibacterial activity from the same donor in our work on kanamycin B analogs. Compound **23** was subjected to Staudinger reaction, hydrogenation, and ion-exchange. The final product, **RT501** (α/β = 7/1) was obtained as a chloride salt. The improved activity of **RT501** against *E. coli* (TG1) (pTZ19U-3), which contain plasmid encoded for APH(3’)-I, demonstrates the efficacy of dideoxygenation at 3’ and 4’ position against APH(3’) (Table IV).

From the MIC values, all the synthesized aminoglycoside with the attachment of AHB group at N-1 regain the activity against both resistant strains of bacteria (Table V). One of the synthesized kanamycin analog, **JLN027**, is even more active than the clinically used amikacin against APH(3’)-I.

Conclusion

Despite of their known high cytotoxicity, aminoglycosides remain to be a valuable resource against formidable pathogens, such as *Pseudomonas aerugenosa*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, and *Enterococcus faecalis*, and their importance has be corroborated by the re-emerging interest documented in the literature. Lessons, which we have learned from our work in pyranmycin and kanamycin aminoglycosides, amazes us that nature has evolved so many effective designs of aminoglycosides, such as the

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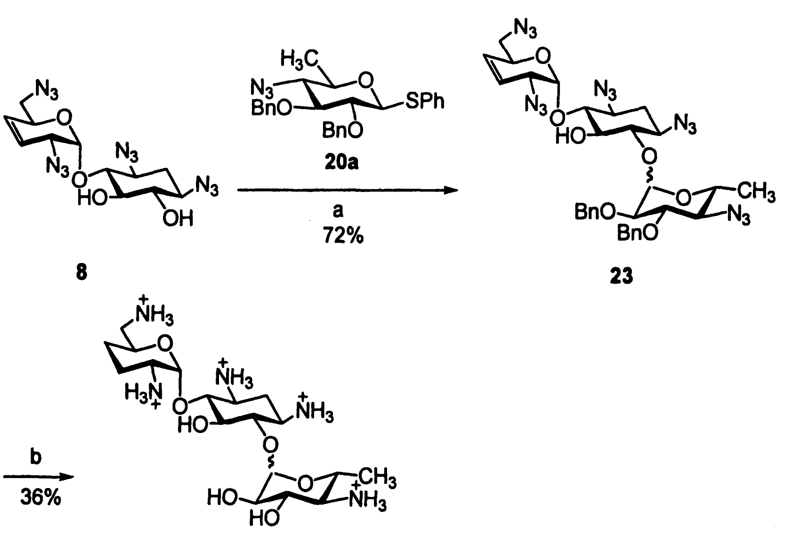

RT501

Conditions: (a) NIS, TfOH, $Et_2O:CH_2Cl_2$ (3:1), (b) (1) PMe_3, NaOH, THF, (2) $Pd(OH)_2/C$, HOAc, H_2O, (3) Dowex 1X8 (Cl^- form).

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Scheme 7. Synthesis of 3',4'-DideoxyKanamycin B Analog

Table IV. Minimum Inhibitory Concentration of the Synthesized Aminoglycoside[a]

Compounds	Strains		
	E. coli (TG1)	*E. coli* (TG1) (pSF815)[b]	*E. coli* (TG1) (pTZ19U-3)[c]
Amikacin	1	1	0.5
Kanamycin B	4	Inactive	32
RT501	8	Inactive	4

[a]: Unit: µg/mL; [b]: plasmid encoded for AAC6'/APH2"
[c]: plasmid encoded for APH(3')-I

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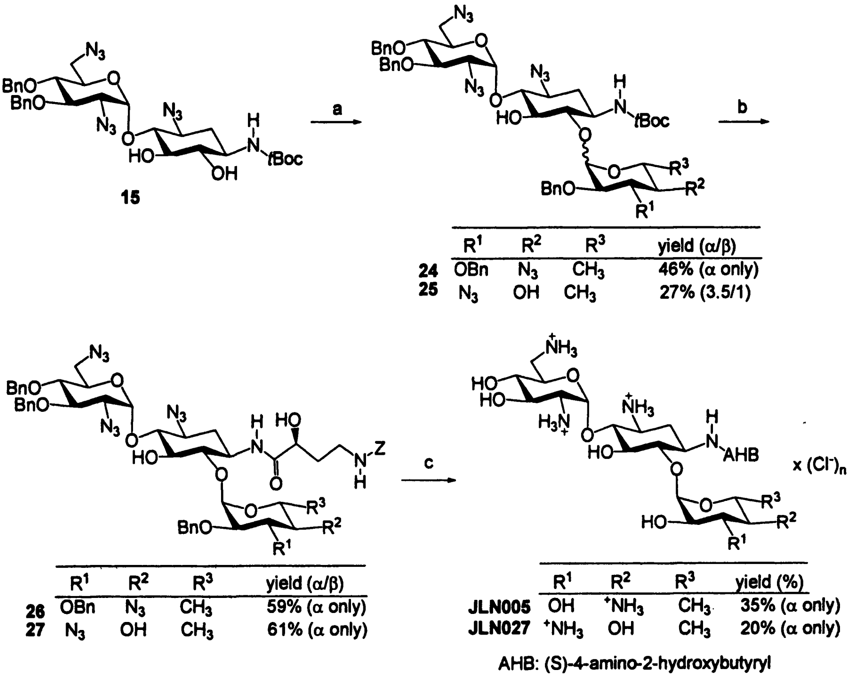
Table V. Minimum inhibitory concentrations of the Synthesized Aminoglycosides[a]

Compounds	Strains		
	E. coli (TG1)	*E. coli* (TG1) (pSF815)	*E. coli* (TG1) (pTZ19U-3)
Amikacin	1	1	0.5
Kanamycin	4	Inactive	Inactive
JLN005	4	2	2
JL005[b]	8	Inactive	Inactive
JLN027	1	1	0.25
JL027[b]	2	Inactive	Inactive

[a]: Unit: μg/mL; [b]: The corresponding kanamycin analogs without AHB group at N-1



	R^1	R^2	R^3	yield (α/β)
24	OBn	N$_3$	CH$_3$	46% (α only)
25	N$_3$	OH	CH$_3$	27% (3.5/1)

	R^1	R^2	R^3	yield (α/β)
26	OBn	N$_3$	CH$_3$	59% (α only)
27	N$_3$	OH	CH$_3$	61% (α only)

	R^1	R^2	R^3	yield (%)
JLN005	OH	$^+$NH$_3$	CH$_3$	35% (α only)
JLN027	$^+$NH$_3$	OH	CH$_3$	20% (α only)

AHB: (S)-4-amino-2-hydroxybutyryl

Conditions: (a) for **24** : phenylthioglycoside, NIS, TfOH, for **25**: glycosyl trichloroacetimidate, BF$_3$-Et$_2$O, CH$_2$Cl$_2$ then NaOMe, MeOH; (b) (1) TFA, CH$_2$Cl$_2$, (2) for **26**: *N*-Cbz AHB, DCC, *N*-hydroxylsuccinamide, THF, Et$_3$N, for **27**: *N*-Cbz AHB, EDC, HOBt, DMF, Et$_3$N; (c) (1) PMe$_3$, THF, H$_2$O, (2) H$_2$, Pd(OH)$_2$/C, HOAc/H$_2$O (1/4), (3) Dowex 1X8 (Cl$^-$ form).

Scheme 8.

3'-deoxygenation and the attachment of AHB group at N-1, long before chemists do. However, synthetic strategies as we have presented here may provide us opportunities of tempering the leads from nature. Perhaps, a semi-synthetic approach, coped with the glycodiversification strategy, will shed light in the battle against resistant bacteria. Developing broad spectrum aminoglycoside antibiotics against a wide range of resistant bacteria is undoubtedly a challenging ordeal. Nevertheless, the merit of synthesizing real compounds for studying their antibacterial activity is irreplaceable. By combining the synthetic achievements with the advancements in aminoglycoside resistance, structural information of the targeted rRNA, bacterial uptake of aminoglycoside, improved administered procedure, we believe that aminoglycoside antibiotics will continue to be a versatile therapeutic for our community.

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Chapter 18

Convergent *N*-Glycopeptide Synthesis

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N-glycopeptides can be synthesized convergently by condensation of glycosylamines with mature aspartic acid containing peptides. Such a union is advantageous when the glycosylamine is particularly precious, or only available in small quantities. However, this approach is also associated with undesired side reactions, *i.e.* aspartimide formation, hydrolysis, and peptide rearrangement, and with the necessity to prepare orthogonally protected peptides. This chapter highlights modern convergent approaches to synthesizing *N*-glycopeptides.

Introduction

N-glycoproteins are ubiquitous on eukaryotic cell surfaces and in body fluids. They constitute a substantial portion of the glycome, the sum of all glycoconjugates and carbohydrate structures in an organism. *N*-glycosylation is a common cotranslational modification of proteins. In *N*-glycoprotein biosynthesis, which takes place at the rough endoplasmatic reticulum, the oligosaccharyltransferase catalyzes the transfer of the tetradecasaccharide $(Glc)_3(Man)_9(GlcNAc)_2$ from the dolichol-linked pyrophosphate donor to an asparagine side chain within the consensus sequence -Asn-X-Ser/Thr- (*1*), whereas X can be any amino acid except for proline (*2*). Upon action by a number of glycosidases and glycosyltransferases the initial *N*-glycan is processed

In Frontiers in Modern Carbohydrate Chemistry; Demchenko, A.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2007.

to afford mature *N*-glycoproteins. *N*-Glycans show large structural diversity with respect to identity and number of monosaccharide units, number of antennae and branching points. The so-called glycoforms account for the microheterogeneity of glycoproteins in biological systems. The glycans of many *N*- and *O*-glycoproteins are known to play critical roles in biological events, e.g. in cellular adhesion processes, immunology, cancer metastasis, and cell growth regulation (*3, 4*). In order to study the structural and functional details of *N*-glycoproteins, their availability in pure form rather than in complex mixtures of glycoforms is needed. These biomolecules can either be isolated from natural sources, or, in principle, they can be prepared by chemical or chemo-enzymatic techniques. While the existence of the aforementioned glycoforms makes the isolation of pure *N*-glycoproteins in sufficient quantities from biomaterials or from recombinant gene technology difficult, the large size and complexity of most *N*-glycoproteins have not yet been mastered by synthetic methods.

In contrast, structurally defined *N*-glycopeptides, which represent partial structures of *N*-glycoproteins, are synthetically accessible in large quantities. They serve as model compounds to gain insight into *N*-glycoprotein structure and function, as tools to study biomolecular interactions and as effectors to evoke biological responses (*5-10*). In addition, libraries of *N*-glycopeptides are synthesized to identify bioactive glycopeptide ligands (*11*). Recently, glycopeptides have experienced a dramatic resurgence partly due to their importance as building blocks for the synthesis of structurally defined glycopeptides and glycoproteins of moderate size by native chemical ligation (NCL). Although NCL is inherently a *convergent* approach, this chapter does not focus on this ligation technique. The topic here is the convergent joining of carbohydrates with peptides, along with the synthetic problems that arise, and how they can be overcome. The reader is referred to the newest literature on *N*-glycopeptide(protein) synthesis by NCL (*12-21*).

There are several established approaches to generating *N*-glycopeptides (*20, 22*). The chemo-enzymatic synthesis of *N*-glycopeptides has the advantage of a considerable reduction of protecting groups, but it requires the availability of glycosyltransferases and nucleoside activated sugar building blocks (*23-27*).

The sequential solid phase peptide synthesis (SPPS) of *N*-glycopeptides by saccharide-compatible Fmoc/*t*Bu strategy, which requires coupling of an Fmoc-protected *N*-glycosyl asparagine building block to the growing peptide chain, is well established (Scheme 1) (*22, 27-33*). However, in order to drive coupling in heterogeneous phase to completion the Fmoc-protected amino acid building block is often applied in 3-5 fold excess, and one faces the dilemma that most Fmoc-*N*-glycosyl asparagine building blocks are very precious. Another concern arises when the carbohydrate moiety of Fmoc-Asn(glycosyl)-OH has unprotected hydroxyl groups, as undesired *O*-acylation can occur during all following coupling steps. Therefore, protection of these hydroxyl groups is advised (17).

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to afford mature *N*-glycoproteins. *N*-Glycans show large structural diversity with respect to identity and number of monosaccharide units, number of antennae and branching points. The so-called glycoforms account for the microheterogeneity of glycoproteins in biological systems. The glycans of many *N*- and *O*-glycoproteins are known to play critical roles in biological events, e.g. in cellular adhesion processes, immunology, cancer metastasis, and cell growth regulation (*3, 4*). In order to study the structural and functional details of *N*-glycoproteins, their availability in pure form rather than in complex mixtures of glycoforms is needed. These biomolecules can either be isolated from natural sources, or, in principle, they can be prepared by chemical or chemo-enzymatic techniques. While the existence of the aforementioned glycoforms makes the isolation of pure *N*-glycoproteins in sufficient quantities from biomaterials or from recombinant gene technology difficult, the large size and complexity of most *N*-glycoproteins have not yet been mastered by synthetic methods.

In contrast, structurally defined *N*-glycopeptides, which represent partial structures of *N*-glycoproteins, are synthetically accessible in large quantities. They serve as model compounds to gain insight into *N*-glycoprotein structure and function, as tools to study biomolecular interactions and as effectors to evoke biological responses (*5-10*). In addition, libraries of *N*-glycopeptides are synthesized to identify bioactive glycopeptide ligands (*11*). Recently, glycopeptides have experienced a dramatic resurgence partly due to their importance as building blocks for the synthesis of structurally defined glycopeptides and glycoproteins of moderate size by native chemical ligation (NCL). Although NCL is inherently a *convergent* approach, this chapter does not focus on this ligation technique. The topic here is the convergent joining of carbohydrates with peptides, along with the synthetic problems that arise, and how they can be overcome. The reader is referred to the newest literature on *N*-glycopeptide(protein) synthesis by NCL (*12-21*).

There are several established approaches to generating *N*-glycopeptides (*20, 22*). The chemo-enzymatic synthesis of *N*-glycopeptides has the advantage of a considerable reduction of protecting groups, but it requires the availability of glycosyltransferases and nucleoside activated sugar building blocks (*23-27*).

The sequential solid phase peptide synthesis (SPPS) of *N*-glycopeptides by saccharide-compatible Fmoc/*t*Bu strategy, which requires coupling of an Fmoc-protected *N*-glycosyl asparagine building block to the growing peptide chain, is well established (Scheme 1) (*22, 27-33*). However, in order to drive coupling in heterogeneous phase to completion the Fmoc-protected amino acid building block is often applied in 3-5 fold excess, and one faces the dilemma that most Fmoc-*N*-glycosyl asparagine building blocks are very precious. Another concern arises when the carbohydrate moiety of Fmoc-Asn(glycosyl)-OH has unprotected hydroxyl groups, as undesired *O*-acylation can occur during all following coupling steps. Therefore, protection of these hydroxyl groups is advised (17).

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Furthermore, Kajihara and coworkers have recently discovered that *N*-glycosylasparagines of complex oligosaccharides can cyclize during the coupling reaction resulting in aspartimides with the glycan attached to the imide nitrogen. This can account for poor incorporation efficiencies even with considerable excess of Fmoc-Asn(glycosyl)-OH.

A popular alternative to the sequential synthesis is the convergent synthesis, in which most often an aspartic acid containing peptide is activated and condensed with a glycosylamine in an ultimate condensation event (Scheme 1) (*5, 10, 18, 19, 34-40*). In solution, the reactants can be utilized in equimolar amounts, or with one component in small excess, which is particularly of interest for glycosylamines available only in limited quantities. Another advantage of the convergent approach is that it allows the synthesis of glycopeptide libraries containing different carbohydrate moieties without the need to resynthesize the peptide for each individual library member. In this chapter, selected examples of successful convergent *N*-glycopeptide syntheses are presented, including the use of complex glycosylamines and challenging peptide sequences.

While the coupling of glycosylamines with *N*-protected aspartic acid esters is usually a straight-forward reaction, this is often not the case for the coupling of glycosylamines with peptides. Besides slow reaction rates, the primary drawback is that persistent byproducts, *i.e.* peptide aspartimides and subsequently peptide rearrangement products can form (Scheme 2) (*15, 41-43*). Not only are these byproducts sometimes the major products, but they can also pose a difficult separation problem. While cyclization occurs easily upon activation of a peptide's aspartyl side chain, aspartimides also form to varying extents when the aspartyl side chain is protected as an ester. The standard protecting group for the aspartyl side chain in Fmoc based SPPS is the *t*-butyl ester. It is less prone to aspartimide formation than the orthogonal allyl ester (*41, 42, 44*).

How easily aspartimides form becomes apparent from the observation that even unprotected aspartic acid and especially asparagine containing peptides undergo this cyclization. Subsequent aminolysis with piperidine at the preferred α- or the β-carbonyl during cleavage of the Fmoc group affords α- and β-piperidites (*42*). Likewise, hydrolysis of the aspartimides affords isoaspartyl peptides as well as aspartyl peptides (Scheme 2) (*45, 46*). For example, the secretory peptide human growth hormone shows a steady increase in isoaspartate formation *in vivo* at pH 7.4 and 37 °C (*47*). Interestingly, aspartimide and isoaspartyl formation in proteins has been linked to protein aging and even to pathogenic processes such as Alzheimer's disease (*48, 49*).

One major factor influencing aspartimide formation upon activation of the aspartic acid's β-carboxyl group is the identity of the neighboring amino acid located *C*-terminal to the aspartic acid. Bodanszky et al. have demonstrated that peptides with Gly, Ala, Ser, Thr, Asn and Glu in this position are especially susceptible to aspartimide formation (*44*). Fields et al. have shown that peptides

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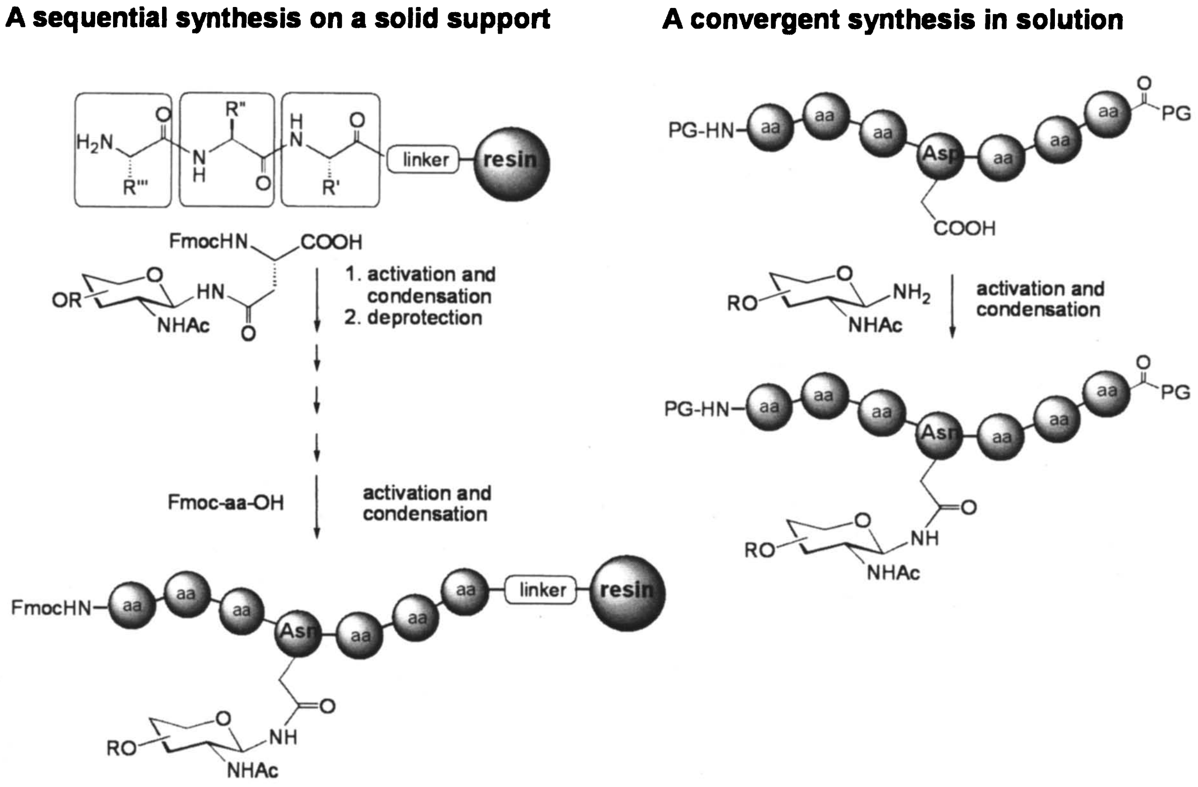
A sequential synthesis on a solid support
A convergent synthesis in solution
H2N
R'''
R''
N H
O
H N
R'
linker
resin
FmocHN
COOH
OR
HN
NHAc
1. activation and condensation
2. deprotection
Fmoc-aa-OH
activation and condensation
FmocHN
aa
Asn
RO
NH
NHAc
PG-HN
Asp
PG
COOH
RO
NH2
NHAc
activation and condensation
PG-HN
Asn
NH
NHAc

Scheme 1

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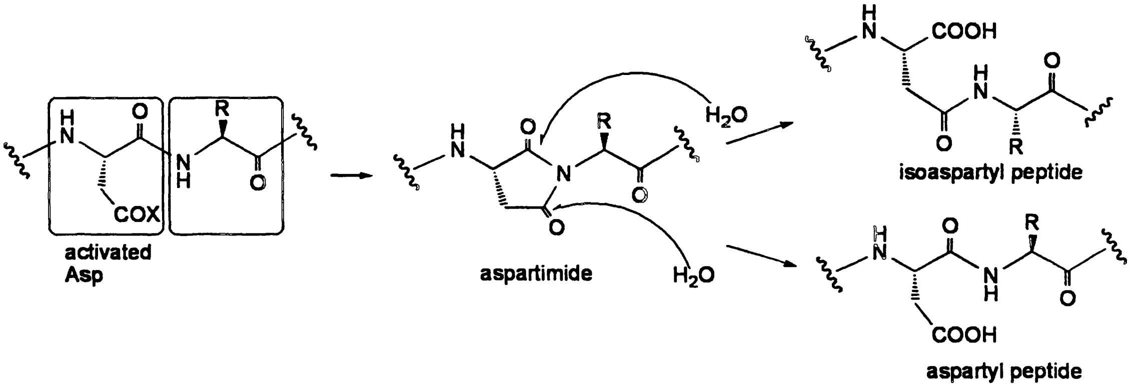
H
N
O
R
N
H
O
COX
activated
Asp
H
N
O
N
R
O
H₂O
aspartimide
O
H₂O
H
N
COOH
H
N
O
O
R
isoaspartyl peptide
H
N
O
R
N
H
O
COOH
aspartyl peptide

Scheme 2

with protected, bulky amino acids such as Arg(Pmc), Asn(Trt), Asp(OtBu), Cys(Am) and Thr(tBu) are also susceptible to this cyclization (*42*). Their study lends support to the notion that aspartimide formation is *inter alia* dependent on the peptide's sequence and its conformation.

Lansbury and coworkers have demonstrated that the amount of aspartimide formed in convergent *N*-glycopeptide synthesis correlates directly with the amount of base present in the reaction mixture (*34, 36*). For example, coupling of the aspartimide-prone peptide **1** and *N*-acetyl-β-glucosaminylamine with 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and N-hydroxybenzotriazole (HOBt) was performed in the absence and in the presence of diisopropylethylamine (DIPEA) (Table 1). While the amount of glycopeptide produced was about 30% in entries 1 and 2, in the absence of DIPEA, 37% of aspartimide and 33% of unreacted peptide **1** was also identified in the product mixture. This is not surprising as 1 equiv. of base is needed for the peptide's deprotonation. In the presence of 1 equiv. DIPEA, the amount of aspartimide increased to 69%, while the amount of unreacted peptide **1** in the product mixture was negligible (entry 2). Using an additional equiv. of glycosylamine instead of DIPEA has a positive effect on the glycopeptide yield (entry 3). Two equiv. of *N*-acetyl-β-glucosaminylamine and 1 equiv. of peptide **1** afforded 55% of the desired *N*-glycopeptide, 45% of the aspartimide, and negligible amounts of **1**. The benefit of using the glycosylamine not only as a nucleophile, but also as a base is most likely a result of its higher concentration and lower basicity when compared to DIPEA.

As a conclusion from Lansbury's detailed study on a number of peptides and glycosylamines, in addition to 1 equiv. glycosylamine, 1 equiv. of a base is needed for a maximum *N*-glycopeptide yield, but at the same time aspartimide formation is also promoted. When the peptide is prone to aspartimide formation, and the glycosylamine is not particularly precious, it is recommended to omit DIPEA, and use 2 equiv. of glycosylamine instead. If the peptide is not susceptible to aspartimide formation, excellent *N*-glycopeptide yields can be obtained with only 1 equiv. of the glycosylamine, and 1-2 equiv. of DIPEA.

An example for a complex glycopeptide synthesis applying the aforementioned protocol is illustrated in Scheme 3. The heptasaccharide-glycosylamine **2** was reacted with peptide **3** in the presence of HBTU, HOBt, and 2 equiv. of DIPEA, which furnished 81% of the *N*-glycopeptide, 12% of peptide **3**, and only 7% of the aspartimide (*36*).

Furthermore, Lansbury's study shows that reaction temperature and reactant concentration have only a small effect on the product ratios. Lowering the temperature or increasing the peptide and/or glycosylamine concentration causes a slight decrease in peptidic aspartimide formation.

Applying conditions similar to the Lansbury protocol an unnatural *N*-glycopeptide bearing a large complex-type diantennary *N*-glycan was convergently synthesized in the Dwek laboratory in the absence of DIPEA (Scheme 4).

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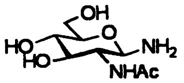
Table I. Dependence of Product Distribution on DIPEA

Entry		Ac-Glu(Fm)Asp-Ala-Ser Lys(Fmoc)-Ala-NH_2 **1**	DIPEA	*N*-glycopeptide	peptide **1**	aspartimide
1	1 equiv.	1 equiv.	-	30%	33%	37%
2	1 equiv.	1 equiv.	1 equiv.	31%	negligible	69%
3	2 equiv.	1 equiv.	-	55%	negligible	45%

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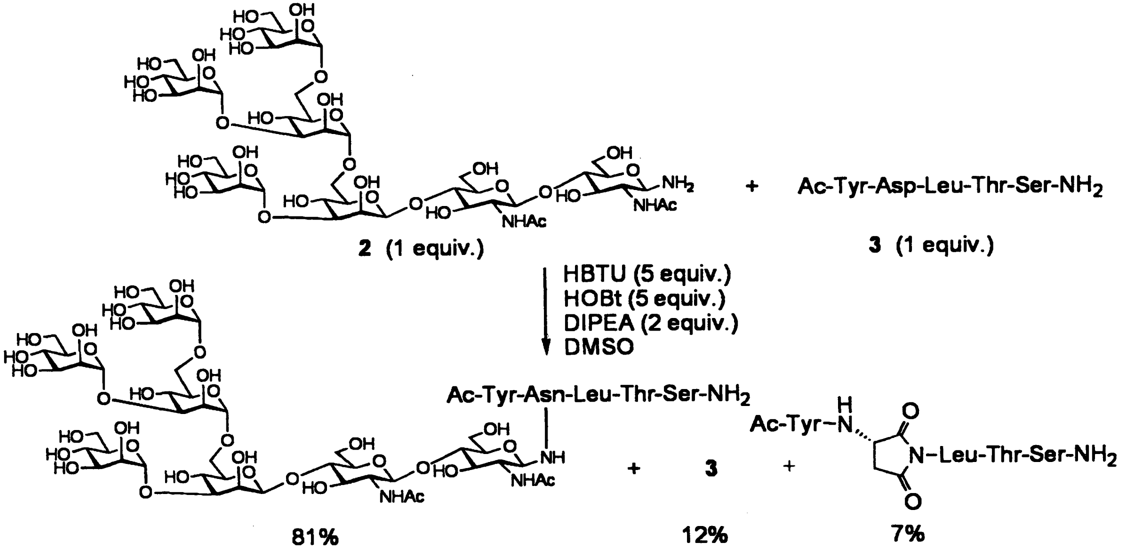
HO
OH
O
NH2
NHAc
2 (1 equiv.)
+
Ac-Tyr-Asp-Leu-Thr-Ser-NH2
3 (1 equiv.)
HBTU (5 equiv.)
HOBt (5 equiv.)
DIPEA (2 equiv.)
DMSO
Ac-Tyr-Asn-Leu-Thr-Ser-NH2
NH
+
3
+
Ac-Tyr-N
H
N-Leu-Thr-Ser-NH2
81%
12%
7%

Scheme 3

In Frontiers in Modern Carbohydrate Chemistry; Demchenko, A.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2007.

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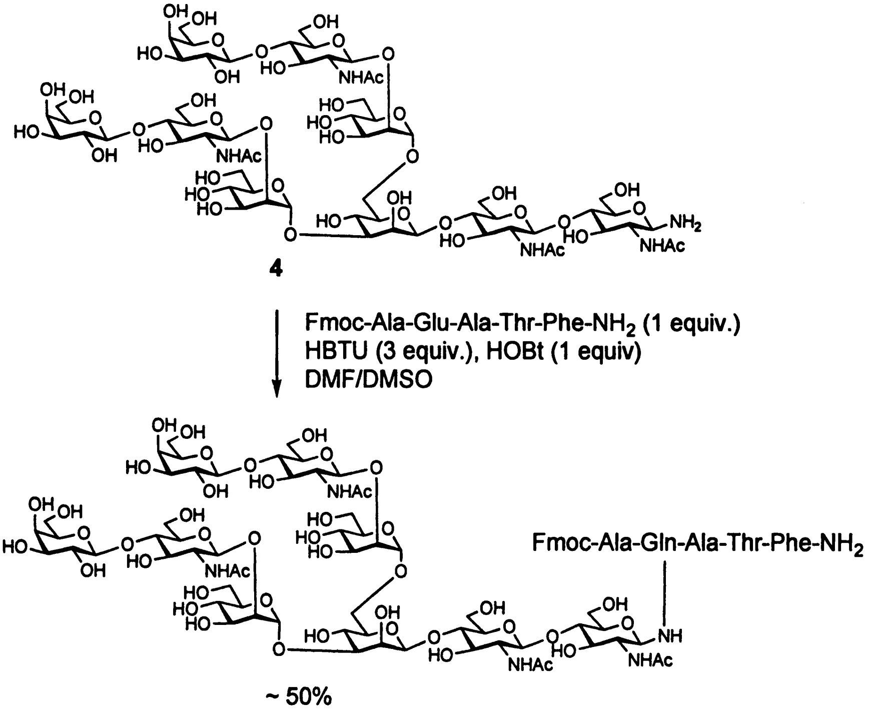
4
Fmoc-Ala-Glu-Ala-Thr-Phe-NH2 (1 equiv.)
HBTU (3 equiv.), HOBt (1 equiv)
DMF/DMSO
Fmoc-Ala-Gln-Ala-Thr-Phe-NH2
~ 50%

Scheme 4

The asialo-nonasaccharide glycosylamine **4** was successfully coupled to the pentapeptide Fmoc-Ala-Glu-Ala-Thr-Phe-NH_2 (equimolar amounts) to afford the glutamine-linked neoglycopeptide in ~ 50% yield (*35*). The condensation of this precious glycosylamine with a peptide containing a glutamic acid residue instead of an aspartic acid residue circumvented the aspartimide problem. In analogy to aspartimides, glutarimides could, in principle, also form. In fact, they have been observed in different contexts (*50*). Luckily, their formation is not as favorable as the formation of aspartimides (*51*).

An example from the Danishefsky group is the synthesis of the complex *N*-glycopeptide **7** (Scheme 5) (*37*). The fully protected pentasaccharide-glycosylamine **5**, which corresponds to the pentasaccharide core common to all *N*-glycans, was constructed from glycal-derived thioglycoside donors and glycal acceptors. The glycosylamine, prepared from the corresponding azide by hydrogenation with Raney-Ni, was substoichiometrically condensed with different peptides, e.g. pentapeptide **6**, in the presence of the coupling reagent 1-isobutyloxycarbonyl-2-isobutyloxy-1,2-dihydroquinoline (IIDQ). A yield of 51% over two steps (reduction and coupling) was achieved. Global deprotection afforded an epimeric mixture of unprotected *N*-glycopeptides **7** quantitatively.

Unfortunately, partial anomerization of the glycosylamine led to an α/β-mixture of the *N*-glycopeptide in a 1:2 ratio.

The production of anomeric mixtures of glycosylamines that were prepared by catalytic hydrogenation of glycosylazides has been previously reported (*52, 53*). The glucosylamines produced had predominantly β-configuration regardless of the substituent at position 2 being neighboring group participating or not. The crude product mixtures of some glycosylamines with protected hydroxyl groups can be recrystallized or chromatographed after hydrogenation of the glycosylazide (*54, 55*). Purified $Ac_3GlcNAc-NH_2$, for example, can does not anomerize in deuterated DMSO or chloroform in several days, which lends support to the idea that the anomerization discussed above takes place during hydrogenation.

Fraser-Reid and coworkers developed a convergent strategy for the synthesis of small *N*-glycopeptides, in which the intermediate generation of the sensitive glycosylamine is avoided (*56*). For example, 2-phthalimido-pentenylglycoside **8** reacts with acetonitrile in the presence of the promoter *N*-bromosuccinimide (NBS) to give a β-configured nitrilium ion, which is trapped by the dipeptide Cbz-Asp-Ile-OMe. The initial addition product rearranges to imide **9** (Scheme 6). Deacetylation can be affected with piperidine in order to establish the authentic β-glycosyl-Asn linkage.

In recent years, the Danishefsky group has favored *O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HATU) as a coupling reagent, e.g. for the convergent synthesis of *N*-glycopeptides bearing *N*-glycans of the complex di-, tri- and tetraantennary type (*19*), the high mannose-type (*18*), as well as the hybrid-type (*10*). The *N*-glycans were synthetically assembled, deprotected and converted into the glycosylamines by Kochetkov amination. This strategy for the installation of an anomeric β-amine does not seem to suffer from anomerization, at least not to the extent previously observed when anomeric azides were hydrogenated (*37*). In fact, Kochetkov amination of a core pentasaccharide analog followed by coupling with a pentapeptide resulted in a single glycopeptide with an anomeric β-configuration (data not shown) (*15*).

Scheme 7 illustrates the convergent synthesis of a prostate specific antigen *N*-glycopeptide bearing a multibranched complex-type tridecasaccharide (*19*). The oligosaccharide precursor (not shown) was synthesized with high stereoselection and maximum convergency. The tridecasaccharide glycosylamine and the partially protected hexapeptide **10** were coupled with HATU and DIPEA in DMSO affording the *N*-glycopeptide with an *N*-terminal cysteine. The protecting groups were removed, and the glycopeptide was subjected to NCL with a pentadecapetide-α-thioester at pH 7.4 to afford the tridecasaccharide-heneicosapeptide **11**. The NCL step gave 65% yield. It should be pointed out that despite remarkable successes NCL, at this point, is not yet ready for the routine ligation of complex glycopeptides (*10, 18*).

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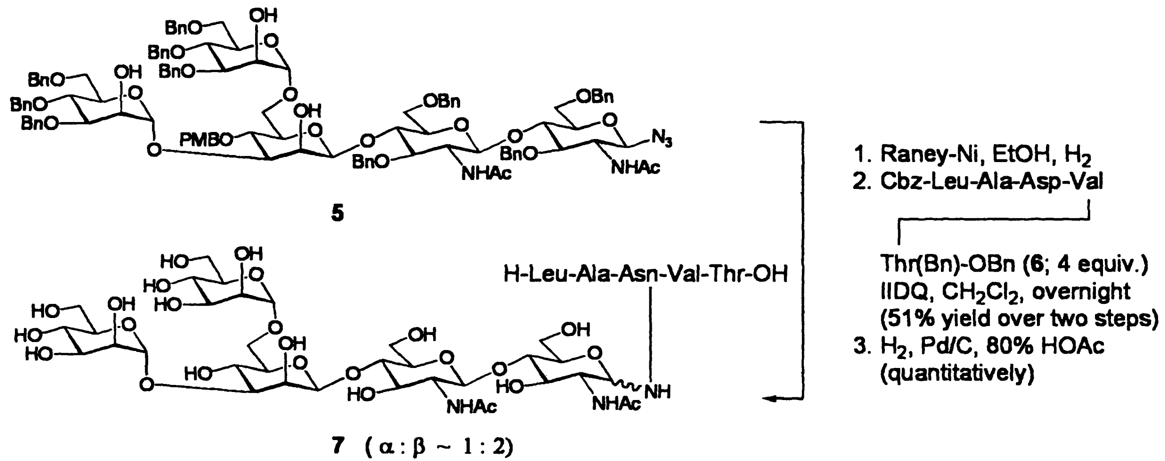
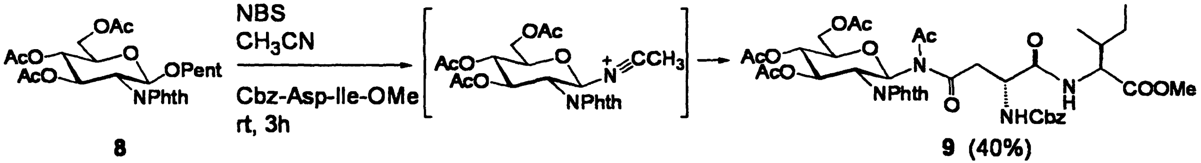

5
1. Raney-Ni, EtOH, H_2
2. Cbz-Leu-Ala-Asp-Val
Thr(Bn)-OBn (6; 4 equiv.)
IIDQ, CH_2Cl_2, overnight
(51% yield over two steps)
3. H_2, Pd/C, 80% HOAc
(quantitatively)
H-Leu-Ala-Asn-Val-Thr-OH
7 (α : β ~ 1 : 2)

Scheme 5


8
NBS
CH_3CN
Cbz-Asp-Ile-OMe
rt, 3h
9 (40%)

Scheme 6

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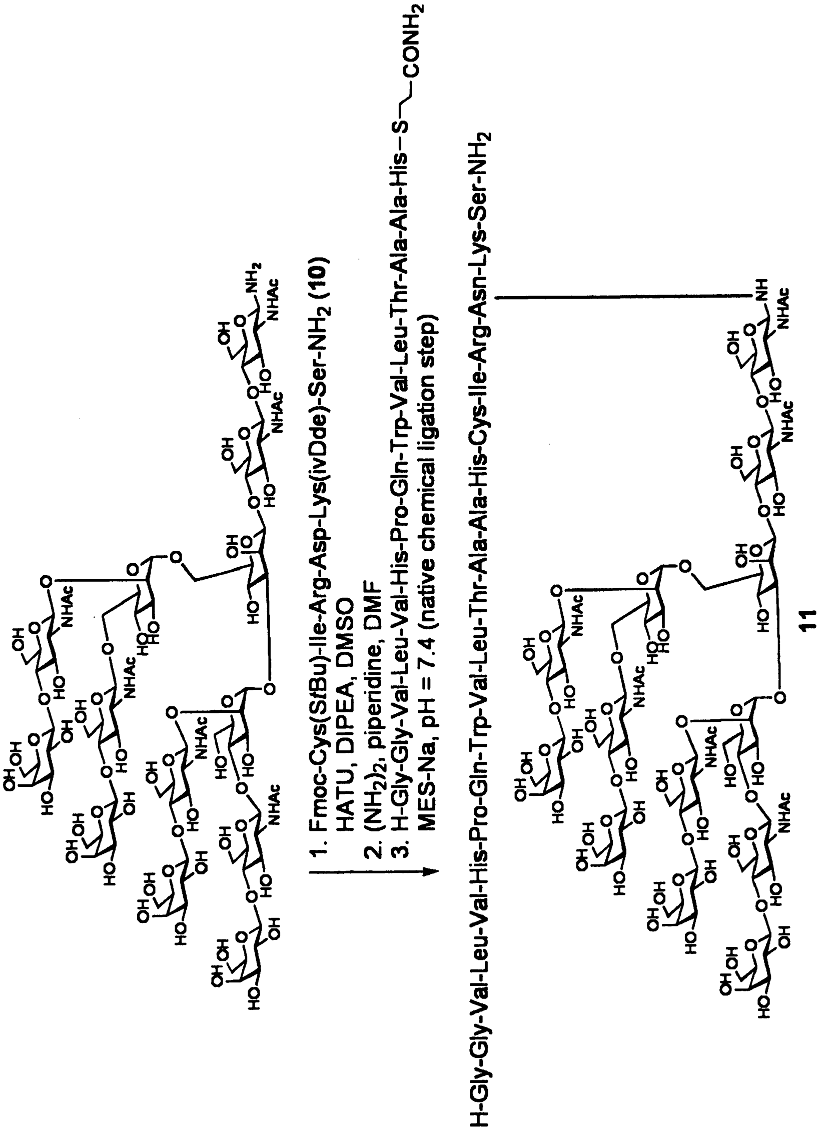
1. Fmoc-Cys(StBu)-Ile-Arg-Asp-Lys(ivDde)-Ser-NH2 (10)
HATU, DIPEA, DMSO
2. (NH2)2, piperidine, DMF
3. H-Gly-Gly-Val-Leu-Val-His-Pro-Gln-Trp-Val-Leu-Thr-Ala-Ala-His-S-CONH2
MES-Na, pH = 7.4 (native chemical ligation step)
H-Gly-Gly-Val-Leu-Val-His-Pro-Gln-Trp-Val-Leu-Thr-Ala-Ala-His-Cys-Ile-Arg-Asn-Lys-Ser-NH2
11

Scheme 7

In order to capitalize on the advantages of solid support chemistry, different on-bead techniques for convergent *N*-glycopeptide synthesis have been developed (*40, 57-61*). Two repeatedly reported strategies are: a) solid phase synthesis of a peptide that contains an orthogonally protected aspartic acid, followed by deprotection and condensation with a pre-prepared glycosylamine; and b) sequential solid phase synthesis of an oligosaccharide on a solid support followed by its conversion into a glycosylamine, and coupling with a pre-prepared aspartic acid containing peptide.

As a first example for the condensation of a resin-bound peptide with a glycosylamine, Johnson's synthesis of a challenging *N*-glycohexapeptide bearing a chitobiosyl moiety is illustrated in Scheme 8. A special feature of this particular approach is a backbone protecting group that effectively prevents aspartimides from forming (*60, 62*).

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Aspartimide formation requires the presence of a nucleophilic amide nitrogen proximal to the aspartic acid at its *C*-terminal side. When this amide is *N,N*-disubstituted, as is the case for the sequence -Asp-Pro-, aspartimides do not form (*42*). The sequence [-Asn(*N*-glycan)-Pro-], however, is not present in eukaryotic *N*-glycoproteins,(*2*) and therefore glycopeptides with this unnatural motif may be of limited use as model compounds. Johnson and coworkers developed an *N*-alkyl backbone-protecting group that is especially attractive for the convergent synthesis of aspartimide-prone peptide sequences. Protection with the *N*-(2-acetoxy-4-methoxybenzyl) group (AcHmb) results in a *N,N*-disubstituted amide, which exhibits a pseudo-proline effect. It is compatible with the Fmoc/*t*-Bu protocol for SPPS, and with the presence of glycosidic linkages. An additional advantage is that *N,N*-disubstituted amide bonds improve a (glyco)peptide's solubility by interrupting aggregation. Application of the AcHmb group in glycopeptide synthesis requires acetylation of the Hmb group and its deacetylation before global deprotection with TFA. These additional synthetic steps may lower the overall reaction yield.

An alternative on-bead strategy was developed in Danishefsky's group (*40, 59, 61*). A glycosylamine is synthesized on a solid support followed by coupling with an aspartic acid containing peptide. Linear or branched oligosaccharides can be constructed by the glycal assembly method (*61*). Scheme 9 shows the synthesis of an unnatural *N*-glycopentapeptide bearing a branched blood group antigen.

After construction of disaccharide **12** with a terminal glucal, the critical terminal GlcNAc-NH_2 residue was installed by azasulfonamidation. Initially, the resin-bound oligosaccharide-glycal reacts with iodonium bis(sym-collidine) perchlorate and anthracene sulfonamide to produce the trans-diaxial iodosulfonamide **13**. Upon treatment with tetrabutylammonium azide the sulfonamide residue migrates from the anomeric center to C-2 replacing the

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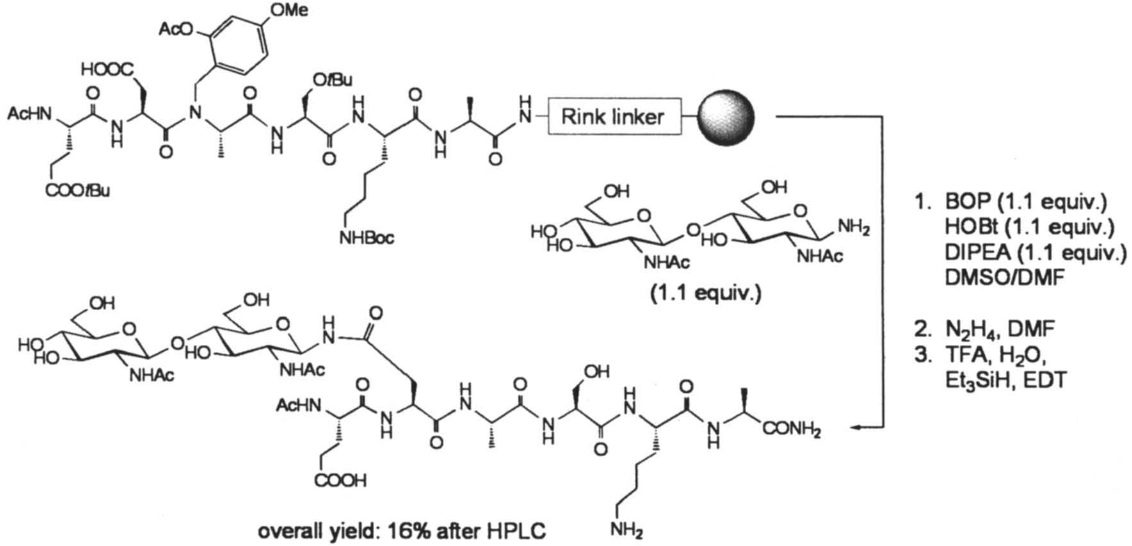
AcHN
COOtBu
HOOC
AcO
OMe
OtBu
NHBoc
Rink linker
OH
HO
NHAc
NH2
(1.1 equiv.)
1. BOP (1.1 equiv.)
HOBt (1.1 equiv.)
DIPEA (1.1 equiv.)
DMSO/DMF
2. N2H4, DMF
3. TFA, H2O,
Et3SiH, EDT
AcHN
COOH
CONH2
NH2
overall yield: 16% after HPLC

Scheme 8

In Frontiers in Modern Carbohydrate Chemistry; Demchenko, A.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2007.

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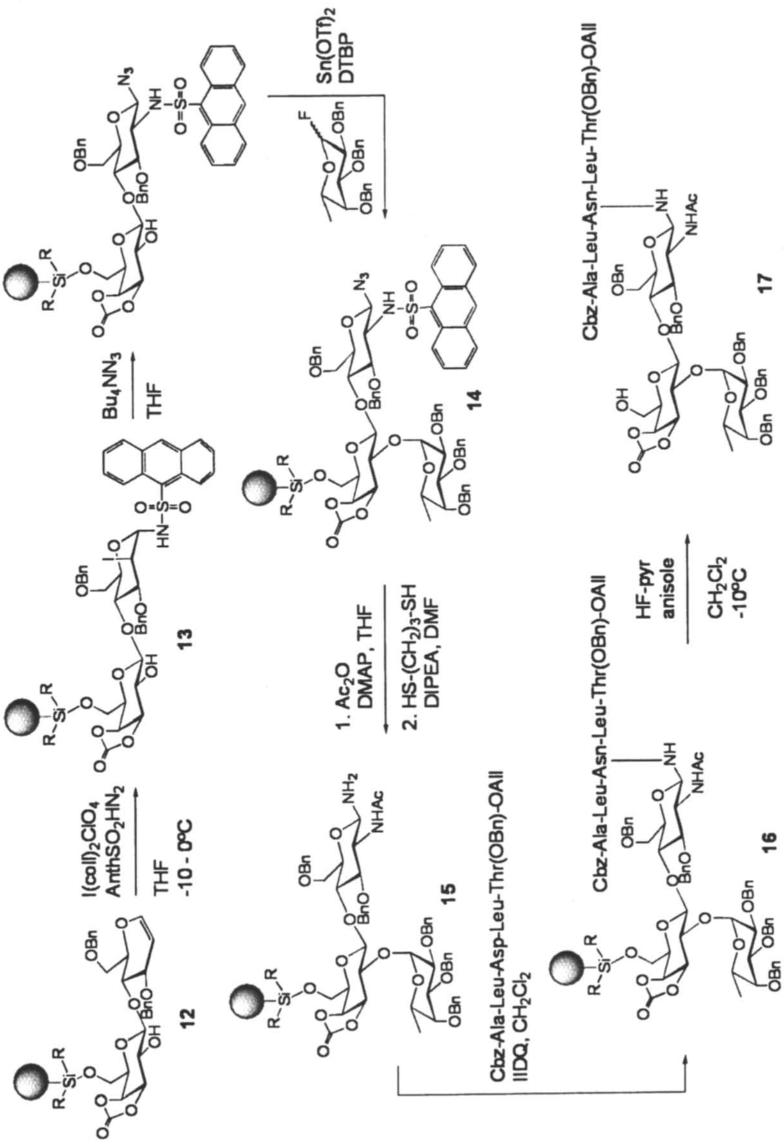
12
I(coll)$_2$ClO$_4$
AnthSO$_2$HN$_2$
THF
-10 - 0ºC
13
Bu$_4$NN$_3$
THF
Sn(OTf)$_2$
DTBP
14
1. Ac$_2$O
DMAP, THF
2. HS-(CH$_2$)$_3$-SH
DIPEA, DMF
15
Cbz-Ala-Leu-Asp-Leu-Thr(OBn)-OAll
IIDQ, CH$_2$Cl$_2$
16
Cbz-Ala-Leu-Asn-Leu-Thr(OBn)-OAll
HF-pyr
anisole
CH$_2$Cl$_2$
-10ºC
17
Cbz-Ala-Leu-Asn-Leu-Thr(OBn)-OAll

Scheme 9

iodide, and an anomeric β-azido group is installed. Fucosylation at the unprotected OH-2 of the galactosyl unit generated the protected trisaccharide **14**. Acetylation of the nitrogen atom of the sulfonamide and subsequent deprotection and reduction resulted in β-glycosylamine **15** with a 2-acetamido substituent. This compound was coupled with a pentapeptide using IIDQ to afford the resin-bound *N*-glycopeptide **16**. Cleavage from the resin with HF-pyridine provided the fully protected glycopeptide **17** in 10% overall yield. Partial defucosylation due to the acid exposure may have lowered the yield.

All the examples of convergent *N*-glycopeptide syntheses discussed so far have the necessity to synthesize orthogonally protected peptides in common. The carboxyl group of interest requires a semi-permanent protecting group during SPPS followed by its selective removal and activation. In Danishefsky's work, the aspartic acid side chain is often protected as a *p*-methoxybenzyl ester (*40*). Vetter, Gallop and coworkers protected aspartic acid or glutamic acid side chains of enkephalin derived peptides as allyl esters (Scheme 10).

The peptides were generated by SPPS on highly acid-labile SASRIN-resin, followed by Pd(0)-catalyzed cleavage of the allyl group (*63*) with an Fmoc-compatible mixture of *N*-methyl-morpholine, acetic acid and chloroform (*64*). The carboxylic acid side chains were converted into their pentafluorophenyl activated esters. A number of unprotected glycosylamines of varying complexity and charge, prepared by Kochetkov amination (*65*), were then condensed with the resin-bound peptides in the presence of HOBt and DIPEA. Fmoc-deprotection and side chain deprotection with simultaneous cleavage from the resin using 50% TFA in dichloromethane afforded the unprotected *N*-glycopeptides. In all cases, the stereochemistry of the anomeric center of the glycosylamines was retained in the glycopeptides, and based on a detailed structural study of a representative *N*-galactopeptide it is believed that aspartimide and isoaspartyl peptide formation was sufficiently suppressed. One factor may be that the sequence -Asp-Tyr(tBu)- is not particularly susceptible to aspartimide formation.

This on-resin ligation is exemplified by means of *N*-glycopeptides **18** and **19** (Scheme 10). Glycopeptide **19** carries a lacto-*N*-fucopentaose II moiety, which is in actuality an *O*-glycan found in human milk. The reaction yields with small uncharged monosaccharide-glycosylamines (not shown) were typically quantitative. Non-fucosylated oligoglycopeptide yields range 50-80%. The illustrated glycopeptide **19** contains an acid-sensitive fucose moiety, which led to 50% product loss due to defucosylation. Glycopeptide **18** shows that even charged glycosylamines can be incorporated, albeit with lower efficiency of 30-50%.

While orthogonal protecting groups for the aspartic acid side chain are available (*40, 63, 66*), at least two synthetic steps are required for deprotection, activation and coupling, which potentially lowers overall reaction yields. The

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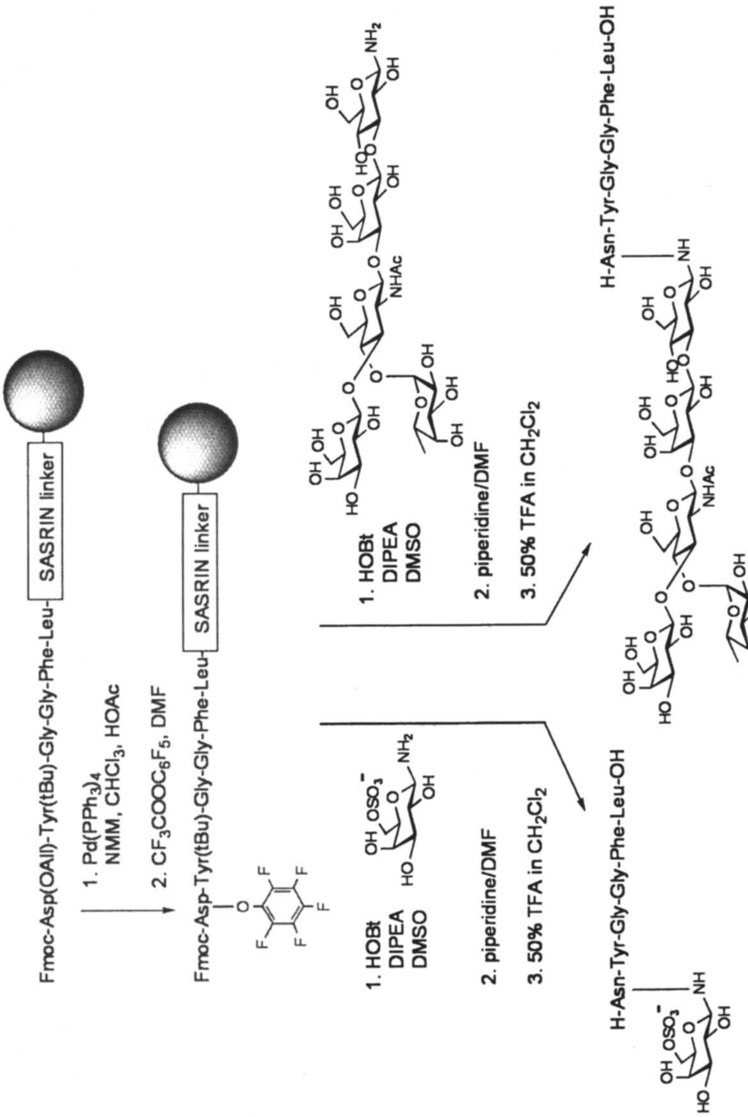
Fmoc-Asp(OAll)-Tyr(tBu)-Gly-Gly-Phe-Leu-
SASRIN linker
1. Pd(PPh3)4
NMM, CHCl3, HOAc
2. CF3COOC6F5, DMF
Fmoc-Asp-Tyr(tBu)-Gly-Gly-Phe-Leu-
SASRIN linker
F
1. HOBt
DIPEA
DMSO
2. piperidine/DMF
3. 50% TFA in CH2Cl2
HO
OH
OSO3−
NH2
H-Asn-Tyr-Gly-Gly-Phe-Leu-OH
NH
NHAc
H-Asn-Tyr-Gly-Gly-Phe-Leu-OH

18

19

Scheme 10

process could be simplified if the protecting group of the aspartic acid side chain were easily converted into an activating group *in situ*, followed by immediate interception of the activated species by a glycosylamine to form the desired amide bond. At the same time, the problem of aspartimide formation and subsequent peptide rearrangement must be addressed. Previously discussed examples have shown that performing the coupling under neutral reaction conditions helps to minimize aspartimide formation. A new solution to both problems, aspartimide formation and multiple step conversion of the aspartyl side chain into a reactive acylation species, is the installation of a photoreactive 7-nitroindoline amide at the aspartyl side chain. The 5-bromo-7-nitroindoline (Bni) moiety efficiently protects the aspartic acid side chain during SPPS (*67*). The latent *N*-acyl-Bni group becomes a powerful acylating species upon excitation with near UV light, which can be exploited for the acylation of glycosylamines.

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Thirty years ago Patchornik and coworkers discovered the unique acylating properties of *N*-acyl-7-nitroindolines (*68, 69*). Since then, only a few applications, usually involving the acylation of amine nucleophiles, have been published (*70-75*). For example, *N*-glycosyl amino acids were efficiently synthesized by phototransamidation (*74, 75*). How does this photoacylation work? Upon excitation in aprotic organic solvents (e.g. dichloromethane, THF, acetonitrile, or DMSO) the *N*-acyl-7-nitroindoline is believed to rearrange to a nitronic anhydride, which, in its anti-conformation (*76*), reacts with an amine to afford an amide as well as recyclable 7-nitroindoline (Scheme 11) (*77, 78*). Water cannot be used as a solvent, as a photoredox reaction takes place instead of an acylationn (*79*).

We have recently demonstrated the photochemical convergent approach to *N*-linked glycopeptides on model sequences of human erythropoietin (hEpo) (*67*), a glycoprotein hormone with three *N*-glycosylation sites at the positions 24, 38, and 83 (*80*). The photoreactive hEpo peptides were synthesized by SPPS with the Fmoc/tBu strategy on extremely acid sensitive Sieber amide resin (*81*). The photoreactive building block Fmoc-Asp(Bni)-OH is readily available from Fmoc-Asp(Bni)-OAll (*75*) by $Pd(PPh_3)_4$ catalyzed deallylation in the presence of *N*-methyl aniline (*82*). Cleavage from the resin with dilute trifluoroacetic acid produced the fully protected photoreactive peptide amides, in ~ 80% yield. As an example, Scheme 12 illustrates the photochemical convergent synthesis of *N*-glycopeptide **21**, and the complex-type *N*-glycopeptides **22** and **23**. Irradiation of peptide **20** in the presence of 2 equiv. of 3,4,6-tri-*O*-acetyl-*N*-acetyl-β-glucosaminylamine, without any additives, gave **21** in an excellent yield of 84%. This result is surprising, as acetylated glycosylamines are known to be poor nucleophiles. In fact, an attempt to couple the same glycosylamine to the peptide Ac-Asp-Val-Phe-NH_2 with HBTU/HOBt gave only 10% *N*-glycopeptide yield (*34*). Photoreactive peptide **20** and substoichiometric amounts of the diantennary asialo glycosylamine **4** were irradiated in DMSO in the presence

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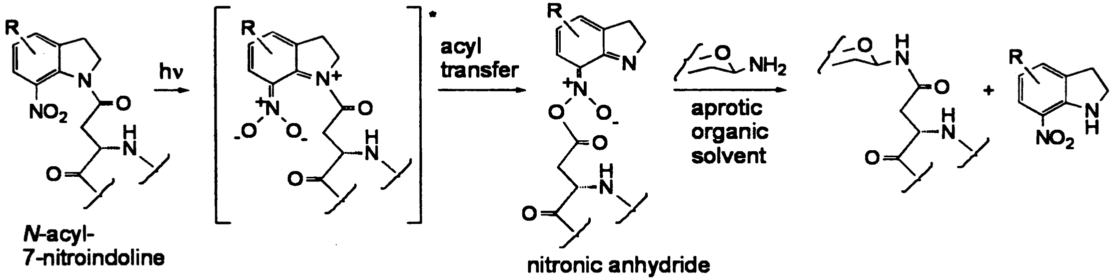
R
N
NO2
O
H
N
O
hν
R
N+
+N
-O
O-
O
H
N
O
*
acyl
transfer
R
N
+N
O
O-
O
H
N
O
O
NH2
aprotic
organic
solvent
O
H
N
O
H
N
O
+
R
N
H
NO2
N-acyl-
7-nitroindoline
nitronic anhydride

Scheme 11

of HOBt (Scheme 12). The desired *N*-glycopeptide **22** was formed at 64% yield. Removal of the two *t*-butyl groups with TFA and the Fmoc group with piperidine gave the fully unprotected *N*-glycohexapeptide **23**.

In summary, as the availability of large and complex *N*-glycans improves, complex *N*-glycopeptides are more often than not synthesized convergently, especially when the oligosaccharide is only available in small quantities. This approach requires the condensation of a glycosylamine with an aspartic acid containing peptide. While unprotected *N*-glycans can relatively easily be converted into β-glycosylamines, the coupling with a peptide can be very inefficient when aspartimide formation dominates. Minimizing the amount of base in the reaction mixture, all the way to neutral coupling conditions adopted from peptide chemistry, helps to increase *N*-glycopeptide yields. Alternatively, incorporation of proline or backbone-protection of the amide-nitrogen located directly *C*-terminal to the aspartic acid completely avoids aspartimide formation. Furthermore, substituting aspartic acid with glutamic acid reduces the amount of intramolecular cyclization.

Regardless of a convergent *N*-glycopeptide synthesis being performed in solution or on a solid support, the synthesis of an orthogonally protected peptide followed by selective deprotection and activation of the aspartic acid side chain is usually necessary. A possibility of shortening this reaction sequence is the preparation of peptides with *o*-nitroindoline protected aspartic acid side chains. These photoreactive peptides can be converted into powerful acylating species by irradiation with light. The *in situ* activated peptides are particularly useful for the acylation of unprotected glycosylamines in the presence of HOBt in DMSO. The photocoupling reaction is conducted in the absence of base, thus reducing the risk of aspartimide formation.

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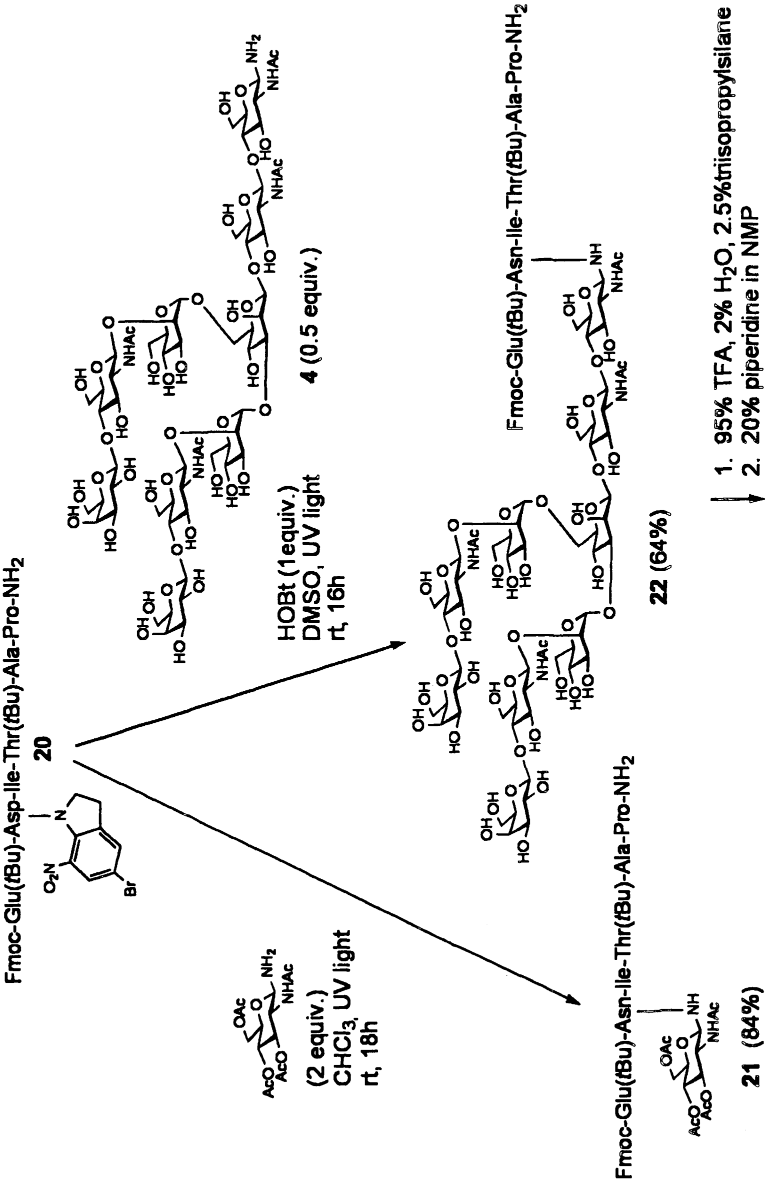
Fmoc-Glu(tBu)-Asp-Ile-Thr(tBu)-Ala-Pro-NH2
20
O2N
Br
(2 equiv.)
CHCl3, UV light
rt, 18h
HOBt (1 equiv.)
DMSO, UV light
rt, 16h
4 (0.5 equiv.)
Fmoc-Glu(tBu)-Asn-Ile-Thr(tBu)-Ala-Pro-NH2
21 (84%)
22 (64%)
1. 95% TFA, 2% H2O, 2.5% triisopropylsilane
2. 20% piperidine in NMP

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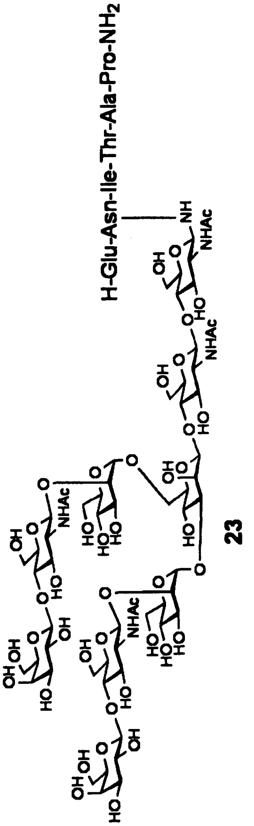
H-Glu-Asn-Ile-Thr-Ala-Pro-NH$_2$
NHAc
NH

23

Scheme 12

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Chapter 19

Targeting Galectin-1 with Self-Assembled Multivalent Pseudopolyrotaxanes

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This review describes the development of self-assembled multivalent pseudopolyrotaxanes as flexible and dynamic neoglycoconjugates for binding Galectin-1 (Gal-1). Gal-1, a dimeric lectin with two lactoside-binding sites, plays multiple roles in a variety of cancers. Pseudopolyrotaxanes comprised of lactoside-displaying cyclodextrin (**LCD**) "beads" threaded onto polyviologen "strings" display highly flexible and adaptable ligands as a result of rotation of the cyclodextrin torus about, and limited translation along, the polymer chain. The pseudopolyrotaxanes rapidly and efficiently precipitate Gal-1 and provide valency-corrected enhancements of up to 30-fold over native lactose and 20-fold over free **LCD** in a T-cell agglutination assay. These results show that the flexible and dynamic ligand presentation afforded by supramolecular assemblies, such as the pseudopolyrotaxanes, is a useful strategy for the study of protein-carbohydrate interactions and the exploitation of multivalency for targeting therapeutically relevant lectins.

In Frontiers in Modern Carbohydrate Chemistry; Demchenko, A.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2007.

Introduction

This chapter presents a summary of a recent and fruitful collaboration between our laboratory and that of Linda Baum in the Department of Pathology at UCLA on a project *(1-4)* that merges supramolecular chemistry *(5)* with glycobiology *(6)* and focuses on multivalency *(7-10)*, a concept that is very much at the heart of both disciplines. Despite extensive investigations, many questions remain to be answered about multivalency: one is the role of flexibility *(11-13)* and adaptability in multivalent binding events. As the individual interactions between lectins (carbohydrate-binding proteins) and their matching carbohydrate ligands are (1) typically quite weak, (2) display fast on- and off-rates, and (3) often take place at the fluid and dynamic interface *(14, 15)* of the cell surface, flexibility and adaptability may play particularly important roles in these multivalent binding events. Recently, we *(1-4)* and others *(16-20)* have used supramolecular chemistry to develop architectures for multivalent binding that are at the flexible and adaptable end of the ligand-display spectrum. In particular, we direct the reader to the excellent work of Yui and co-workers *(16-20)* on *polyrotaxanes* for the multivalent presentation of carbohydrates *(18, 20)* and other ligands *(16, 17, 19)*. Here, we summarize the development *(1-4)* of *pseudopolyrotaxanes* for binding Galectin-1 (Gal-1) *(14, 21-26)*.

Gal-1 is the prototypical member of the galectins *(24, 27-29)*, a ubiquitous family of galactoside-binding lectins that mediate cell adhesion, signaling, and death. Certain galectins, particularly Gal-1 and Galectin-3, are overexpressed in many types of cancer *(27-31)*. Gal-1 plays multiple roles in cancer, from promoting motility and homotypic cell agglutination—which is believed *(27)* to be important for aggregating cancer cells into tumor emboli, thus increasing malignant cell survival in circulation—to defense *(30, 31)* against the immune system by exploiting its natural proapoptotic *(22, 24)* function. Gal-1 is a soluble 14 kDa dimeric galactoside-binding lectin that can organize *(14, 23, 25)* cell surface glycoproteins through binding and crosslinking of terminal or polymeric *N*-acetyllactosamine residues. Synthetic multivalent ligands *(32-36)* for Gal-1 have the potential to act as cancer diagnostics and therapeutics and to shed light on the supramolecular interactions of Gal-1 and its natural and oncogenic carbohydrate ligands. Topologically *(8, 21)*, Gal-1 is a particularly interesting challenge since it is a rigid dimer with two binding sites oriented in opposite directions such that the entrance to each of the binding sites is located 6 nm apart. Chemists targeting Gal-1 have focused primarily on glycodendrimers *(32)* and smaller glycoclusters *(33-36)* with mixed results.

In addition to dendrimers *(32, 37)* and clusters *(33-36, 38, 39)*, two of the main avenues of pursuit by chemists for multivalent display of carbohydrates are neoglycopolymers *(8, 40-43)*, and *dynamic* self-assembled systems *(44-50)* such as liposomes and micelles. Pseudopolyrotaxanes *(1-4)* and polyrotaxanes *(18, 19)* incorporate the advantages of both the polymeric and dynamic approaches.

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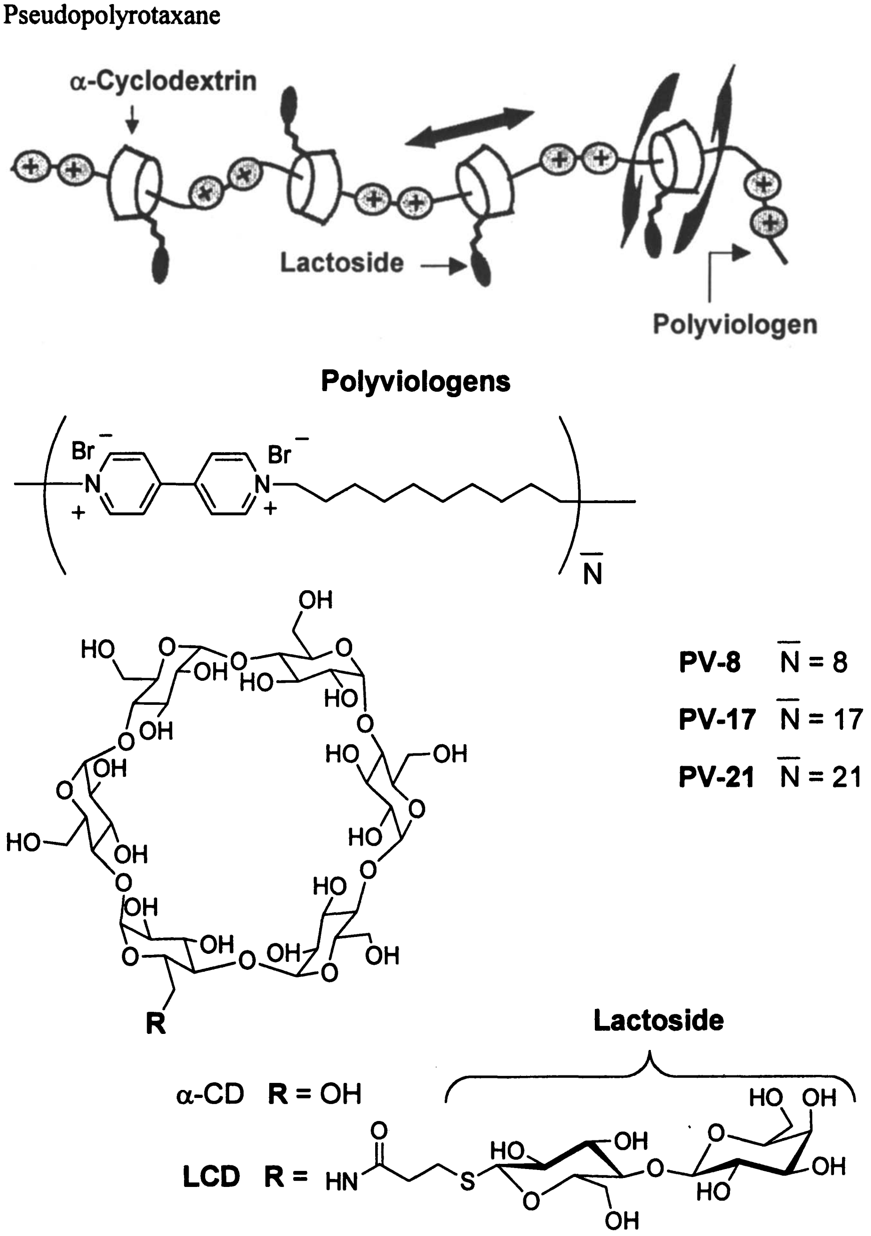
Pseudopolyrotaxane
α-Cyclodextrin
Lactoside
Polyviologen
Polyviologens
Br⁻
Br⁻
N̄
PV-8 N̄ = 8
PV-17 N̄ = 17
PV-21 N̄ = 21
R
Lactoside
α-CD R = OH
LCD R =

Figure 1. Schematic representation of pseudopolyrotaxanes composed of lactoside-cyclodextrin (LCD) rings and polyviologen threads, and the structural formulas of the components.

Pseudopolyrotaxanes *(51)* are supramolecular assemblies that resemble "beads-on-a-string," whereas polyrotaxanes *(52)* are the related mechanically-interlocked molecules in which the string is terminated with bulky stoppers so that the rings cannot escape. In a pseudopolyrotaxane, the rings are able to *thread* and *dethread* on and off the backbone, which is typically a linear polymer, and their binding to the polymer is subject to equilibrium control. Based on the pioneering studies of Harada *(53)* and Wenz *(54)*, our group *(1-4)* and Yui and co-workers *(16-20)* have both used modified cyclodextrins (CDs) *(55-57)* as the ring components of the supramolecular systems. While many of the seemingly exotic structures produced by supramolecular chemists are incompatible with water, the well-known aqueous supramolecular chemistry of CDs *(12, 58-61)* and their inherent biocompatibility *(57)* suggests that they are well-positioned to interface synthetic supramolecular and related mechanically-interlocked architectures with biological systems. Indeed, CD-based (pseudo)polyrotaxanes, which, when carefully designed *(53, 54)*, retain the aqueous solubility of free CDs, are being explored for biological applications *(16-20, 62, 63)*, ranging from drug delivery to tissue engineering. Our interest in developing such systems as *dynamic multivalent neoglycoconjugate*s stems for the fact that, once assembled, the CD rings are able to spin around the axis of the polymer chain as well as move back and forth along the polymer backbone. The extent of these movements can be varied by the choice of the polymer chain *(2)*. If one considers a ligand attached to each CD ring, the position of this ligand is, to a large extent, independent of the position of a ligand on a neighboring ring, for, while the ligands are connected mechanically via the encircled polymer backbone, they are not directly covalently bound to each other. Both ligands will have considerable freedom, including up to 360° of rotation, to respond to a receptor. Thus, pseudopolyrotaxanes offer several potential advantages for multivalent ligand presentation, including (1) the ability to span large distances, (2) the ease of varying ligand densities, (3) their adaptability, and (4) the ease of their synthesis by means of self-assembly. Here, we describe our results *(2, 4)* for Gal-1, the first lectin we have targeted with these supramolecular assemblies.

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Pseudopolyrotaxanes

Initially, we focused *(1, 64)* on pseudopolyrotaxanes composed of lactoside-modified α- and β-CD derivatives threaded onto poly(tetrahydrofuran) and poly(propylene glycol). When 2,6-di-*O*-methlyated CD derivatives were used, soluble complexes were obtained, but the threading and dethreading of the CDs was fast on the ^{1}H NMR time-scale to the extent that isolating the pseudopolyrotaxanes was not possible. We then turned our attention to polycationic polymers *(64)*, in particular, polyviologens. Polyviologen AB-copolymers, comprising alternating decamethylene (A) and positively charged bipyridinium (B) segments, form *(2, 65-69)* stable, water-soluble complexes with α- CD and the lactoside-bearing derivative **LCD**. In aqueous solution, the α-CD rings thread onto the polymer chain and rest predominantly on its decamethylene segments *(2)*, stabilized by the hydrophobic interactions inside the cavities of the CDs. The positive charges, associated formally with the nitrogen atoms on the bipyridinium segments of the polymer, act as electronic "speed bumps" *(54, 65-69)* which reduce the translational motion of the CD rings. By separating the preferred guest into domains flanked by "speed bumps," polyviologens can support high levels of threading at equilibrium (depending on the concentration), with the time-scale *(3)* of self-assembly being slow (3 – 30 days), because of the necessity for the CD rings to have to pass over multiple bipyridinium segments. For example, we found that in the self-assembly *(2)* of pseudopolyrotaxane **[17:17]**, a mixture of 17 equivalents of **LCD** (20 mM) with a polyviologen containing, on average, 17 repeating units (**PV-17**) resulted in over 90% threading. The designation **[17:17]** is meant to reflect the equivalents of ring to thread *in the mixture, not necessarily on polyviologen backbone* : the average number of repeating units in the polyviologen backbone (i.e., 17 for **PV-17**). Stored at this concentration, such mixtures retain the same degree of threading and show no signs of chemical degradation of the components, even after months at room temperature.

Table 1: Pseudopolyrotaxanes

Assembly[a]	LCDs or α-CDs /PV	PV	Expected Threading	TBA %[b] After Self-Assembly[c]	TBA % After Dilution[d]
[17:17]	17	**PV-17**	Over 90%	-	-
α-[17:17]	17	**PV-17**	Over 90%	-	-
[5:21]	5	**PV-21**	One Quarter	23	13
α-[5:21]	5		One Quarter	29	17
[10:21]	10	**PV-21**	Half	51	30
α-[10:21]	10		Half	50	28
[21:21]	21	**PV-21**	Nearly Full	87	52
α-[21:21]	21		Nearly Full	88	56
[42:21]	42	**PV-21**	Nearly Full + Free **LCD**	93	85
[2:8]	2	**PV-8**	One Quarter	45	35
[4:8]	4	**PV-8**	Half	75	47
[8:8]	8	**PV-8**	Nearly Full	97	72

[a] The designation α denotes an pseudopolyrotaxane with native α-CD, all others have **LCD** as the ring component. [b] TBA %, the degree of threading as measured in the Bradford assay, is defined as: 100 x [1 – (OD_{595} sample – OD_{595} background)/(OD_{595} free **PV** – OD_{595} background)]. [c] Self-assembly (3-30 days) of all pseudopolyrotaxanes was carried out at 20 mM in **LCD**. [d] TBA % for the re-established equilibrium (2-4 days) following dilution to 1 mM polyviologen repeating unit.

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Initially, ^{1}H NMR spectroscopy and TLC were used to follow the self-assembly process *(2)*. Later, we discovered *(3)* that the Bradford assay *(70-72)*—a biochemical tool usually highly selective for proteins—could be used to monitor the threading and dethreading of polyviologen-derived pseudopolyrotaxanes. The Bradford assay is a simple colorometric assay based on the dye Coomassie Blue G-250, which undergoes a blue-shift upon binding proteins, such that an increase in absorbance at OD_{595} can be related to the protein concentration. We intended to use the Bradford assay to report on Gal-1 concentration in the precipitate and/or remaining in solution following precipitation experiments with the pseudopolyrotaxanes. During the development *(4)* of this precipitation assay, we noticed *(3)* that the free polyviologens **PV-8** and **PV-21** (average of 8 and 21 repeating units, respectively) yielded a large Bradford response, and even more surprisingly, pseudopolyrotaxanes of **PV-8** and **PV-21** yield correspondingly lower responses based on the expected degree of threading. The correlation was especially good for the **PV-21**-derived assemblies, while the Bradford assay appears to overestimate the degree of threading for the **PV-8**-based assemblies. We introduced *(3)* the TBA ("Threading by Bradford Assay") percentage as a quantitative measure of the degree of threading in such systems. Shown in **Table 1** are TBA percentages at equilibrium for a selection of pseudopolyrotaxanes at the concentration used for self-assembly (20 mM in **LCD** or α-CD) and after dilution to a concentration of 1 mM polyviologen repeating unit. At least by this measure, attachment of the lactoside has little effect the degree of threading at equilibrium, although it does appear to have an effect on the kinetics of assembly and disassembly. As expected for complexes subject to equilibration, all of the pseudopolyrotaxanes tested using the Bradford assay undergo dethreading upon dilution; dethreading, however, is slow to the extent that diluted assemblies are kinetically stable on the time-scale (15 minutes) of the agglutination experiments. Equilibrium was re-established *(3)* within 2 or 4 days (**LCD** and α-CD assemblies, respectively), with noticeable dethreading occurring within 4 hours for assemblies **[21:21]**, **[10:21]**, **[8:8]**, and **[4:8]** (4 hours after dilution the TBA percentages are 73, 39, 83, and 55%, respectively, compare to starting and final values in **Table 1**). The concentration of 1 mM polyviologen repeating unit represents a 20-fold dilution in both **LCD** and **PV-21** for pseudopolyrotaxane **[21:21]**, this yields a TBA of 52% compared at 87% at the concentration of the 20 mM stock solution. By contrast, assembly **[42:21]**, which contains the full extra equivalent of **LCD**, is much more resistant to dethreading.

Gal-1-Induced T-cell Agglutination

We have developed a straightforward assay *(2)* to evaluate the pseudopolyrotaxanes and relevant control compounds based on the ability of Gal-1 to aggregate cells, which is used to malignant advantage *(27)* in cancer. Treatment of CEM cells (cultured human T-leukemia cells) with 10 mM recombinant human Gal-1 *(73)* for 5 min at 37 °C results in large aggregates that resemble tumor emboli (**Figure 2A**). As determined by light microscopy, pretreating Gal-1 with 2 mM lactose (final concentration) for 10 min at room temperature allows the cells to remain largely dispersed, whereas a mixed state of aggregated and dispersed cells is observed down to 1.5 mM lactose. A value of 1.5 mM for the minimum inhibitory concentration (MIC) of lactose with Gal-1 matches well with the literature values in other agglutination assays *(33, 74)*. In addition to lactose itself, polyvalent lactosides, such as the trivalent glycocluster **1** *(2)* (**Figure 3**) were inhibitory, as shown in the titration of depticted in **Figure 2B**. However, on a per-lactoside basis (valency corrected MIC = 1.5 mM), the trivalent cluster **1** is not any better an inhibitor than lactose itself: thus, no multivalent enhancement is detected in this assay. In contrast, a multivalent enhancement was detected for the neoglycopolymer **2** *(2)* (**Figure 3**); this enhancement, at less than 2-fold (valency corrected MIC = 900 μM), is still rather small however.

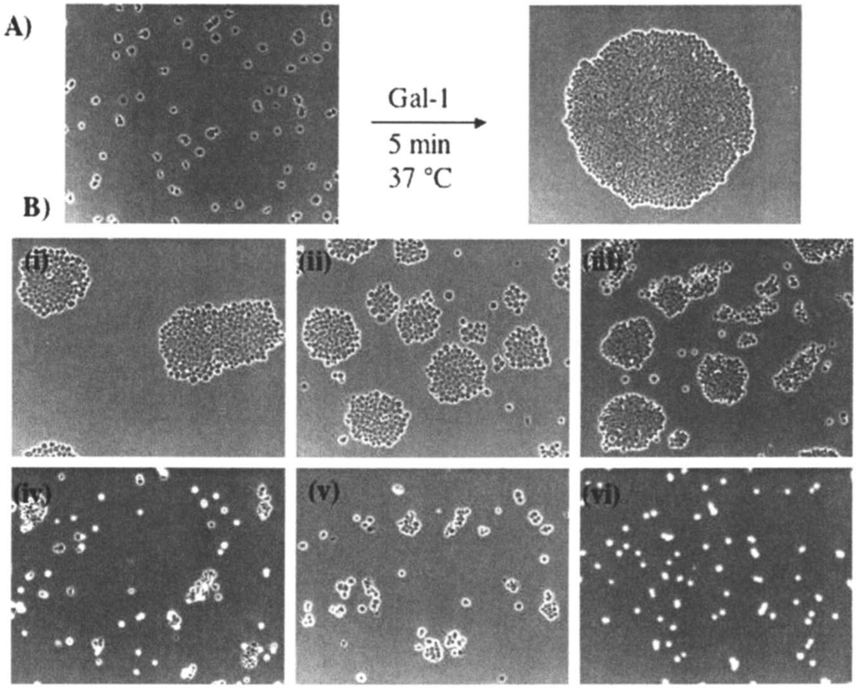

*Figure 2. **A)** Normally dispersed CEM cells are aggregated by Gal-1 (10 μM). **B)** Titration of neoglycocluster 1 in the presence of CEM cells and 10 μM Gal-1: **(i)** at 200 μM 1 cells are aggregated, **(ii)** at 300 μM 1 cells are aggregated, **(iii)** at 400 μM 1 cells are aggregated, **(iv)** at 500 μM 1 cells are in the mixed state of small aggregates and dispersed cells, **(v)** at 600 μM 1 cells are in the mixed state, **(vi)** at 700 μM 1 cells are dispersed.*

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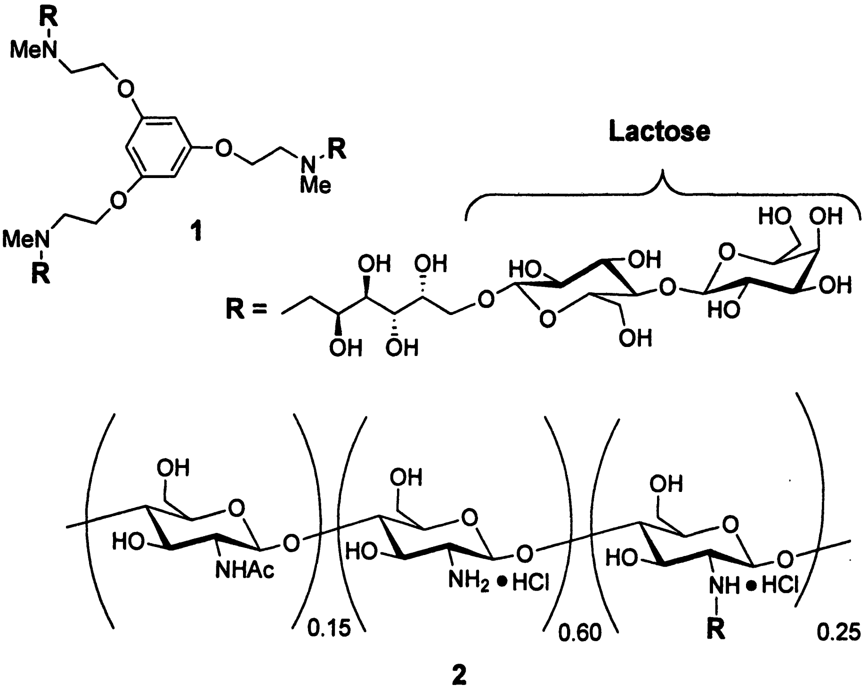

Figure 3. Structural formulas of the trivalent neogylcocluster 1 and the chitosan-derived neoglycopolymer 2.

The neogylcopolymer **2** was derived from chitosan, a linear polysaccharide of repeating β-1,4-linked glucosamines and *N*-acetylglucosamines, which is obtained from the partial hydrolysis of the *N*-acetyl groups of chitin *(75)*. In the neoglycopolymer **2**, 25% of the monosaccharide residues of a commercially available chitosan polymer are substituted with lactoside via the same galactitol linker *(38)* used in the glycocluster **1**. Like cellulose, chitosan possesses a rigid linear conformation on account of the presence of intramolecular hydrogen bonds in its secondary structure. Functionalized chitosan has been investigated by a number of groups for applications such as drug delivery *(76)*, antimicrobial activity *(77)*, and multivalent presentation of carbohydrates *(78)*. Here, chitosan provides a rigid rod scaffold for the *covalent* multivalent display of lactosides.

The small multivalent enhancement observed in the agglutination assay for lactoside displayed covalently from a rigid polymer backbone contrasts with greater enhancements obtained *(2, 4)* for the flexible and adaptable pseudopolyrotaxanes. Interestingly, **LCD** alone displays a 1.5-fold enhancement in efficacy over lactose, with an MIC (1000 μM) close to the valency-corrected MIC of the neoglycopolymer **2**. We attribute this enhancement to a greater

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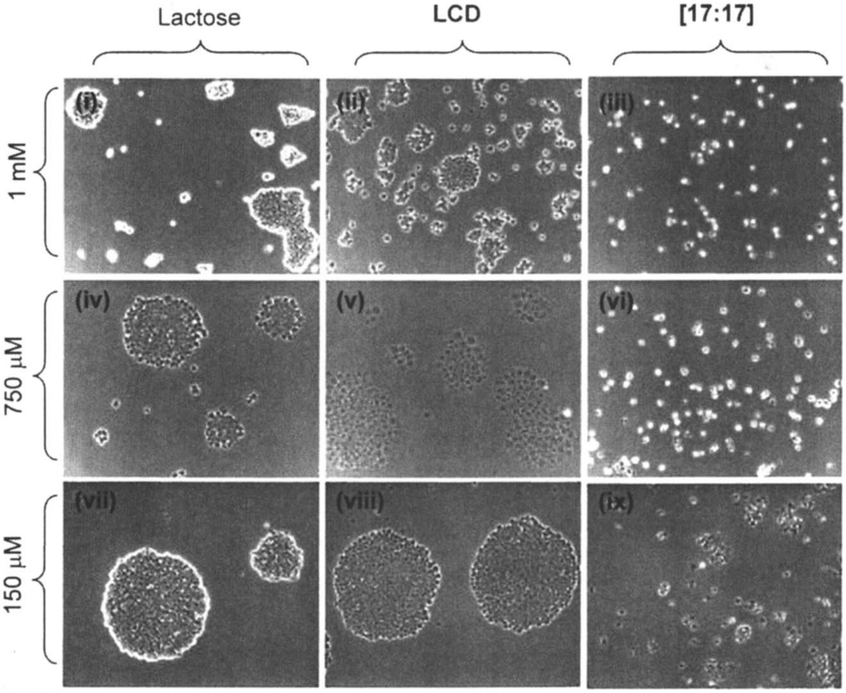

Figure 4. Titrations of lactose, ***LCD****, and pseudopolyrotaxane [17:17] in the presence of CEM cells and 10 µM Gal-1. Valency-corrected lactoside concentrations are shown at the left.* ***(i)*** *at 1 mM lactose cells are aggregated,* ***(ii)*** *1 mM* ***LCD*** *cells are in the mixed state,* ***(iii)*** *1 mM [17:17] cells are in the dispersed state,* ***(iv)*** *at 750 µM lactose cells are are aggregated,* ***(v)*** *at 750 µM* ***LCD*** *cells are aggregated,* ***(vi)*** *at 750 µM [17:17] cells are dispersed,* ***(vii)*** *at 150 µM lactose cells are aggregated,* ***(viii)*** *at 150 µM* ***LCD*** *cells are aggregated,* ***(ix)*** *at 150 µM [17:17] cells are in the mixed state.*

burial of hydrophobic surface upon binding **LCD** versus lactose including non-specific interactions of the CD portion of **LCD** with the surface of Gal-1 near the binding site. We also note that **LCD** is a thio-lactoside with a short alkyl linker, compared to lactose (OH, no linker) or the longer open-chain carbohydrate linkers (O-galactitol) of cluster **1** and polymer **2**. Regardless of the reasons for the enhancement for **LCD** alone, its efficacy is greatly increased by threading unto the polyviologen backbone to yield the pseudopolyrotaxanes. Originally *(2)*, we tested **(Figure 4)** assembly **[17:17]**, which, on a valency-corrected basis, yields the dispersed state down to 750 µM, where lactose and **LCD** yield the aggregated state. Pseudopolyrotaxane **[17:17]** yields the mixed state down to 150 µM, at which point lactose and **LCD** yield extensive aggregation. The MIC of 150 μM represents a valency-corrected enhancement of 10-fold over lactose and 6.7-fold over **LCD**. The concentration of polymer at the MIC is only 8.8 μM, close to the concentration of Gal-1 (10 μM) being inhibited. We also tested free **PV-17** and **α-[17:17]** (pseudopolyrotaxane composed of **PV-17** and 17 equivalents of native α-CD, no lactoside) in the agglutination assay. Neither were inhibitors, indicating that the effects of assembly **[17:17]** are indeed mediated by the lactoside ligand.

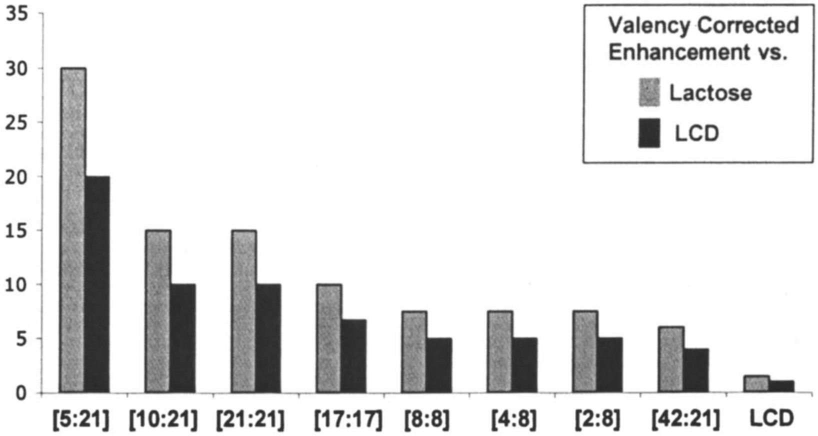

Figure 5. Results of the agglutination assay for a range of pseudopolyrotaxanes presented as a chart of valency corrected enhancements (per-lactoside MIC normalized to MIC for free lactose (grey) or free ***LCD*** *(black).*

We then compared *(4)* pseudopolyrotaxanes self-assembled from longer (**PV-21**) and shorter (**PV-8**) polyviologens using different equivalents of **LCD** (**Table 1**) in the agglutination assay. The multivalent enhancements (valency-corrected MIC) for the full range of pseudopolyrotaxanes tested vs. MIC for free lactose and **LCD** are shown in **Figure 5**. Assembly **[42:21]**, where half of the **LCDs** are not located on polymer backbone, is the worst performer, but it still generates a 4-fold enhancement over free **LCD** or a 6-fold one over lactose. Slightly better enhancements are obtained for the **PV-8**-based assemblies, which each have the same valency-corrected MIC (200 μM). This result represents an enhancement of 7.5-fold vs. lactose and 5-fold vs. **LCD**, i.e., significant gains, at least for the quarter-threaded assembly **[2:8]**, considering there are only two lactoside ligands (on average) connected in this pseudopolyrotaxane. For the nearly fully threaded assemblies there is an interesting progression in the valency-corrected MIC with increasing polyviologen chain length, going from **[8:8]** (200 μM) to **[17:17]** (150 μM) to **[21:21]** (100 μM), which corresponds to multivalent enhancements of 5-, 6.7-, 10-fold vs **LCD**, and 7.5-, 10-, and 15-fold

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vs. lactose, respectively. However, the best performer overall is the quarter-threaded 21-mer **[5:21]** with a valency-corrected MIC of 50 μM. It displays a 30-fold enhancement over lactose and 20-fold over free **LCD**, while it is 2-fold better than the nearly fully threaded assembly **[21:21]**, despite having only one quarter the number of available lactoside ligands. The roughly half-threaded assembly **[10:21]** yields the same MIC (100 μM) as the nearly fully threaded assembly **[21:21]**, confirming that, on a per-lactoside basis, it is not necessary to maximize the number of **LCDs**/thread to attain the mixed state at a low concentration. Indeed, the valency-corrected enhancement for **[5:21]** is the highest we are aware of for inhibiting Gal-1 in a cell-based assay, being even greater than the valency-corrected enhancement (16-fold) observed *(33)* for the glycoprotein asialofetuin. Potent (14.5-fold) multivalent enhancement in cell-based assays for Gal-1 inhibition has also been obtained for wedge-shaped glycodendrons synthesized by Pieters and co-workers *(33)*. However, these enhancements are significantly lower than those that have been obtained in solid-phase competition assays, where spectacular multivalent enhancements for Gal-1 inhibition have been observed by Gabius and co-workers (*32-36*), in particular for the Pieters glycodendrons (*33*) and the starburst glycodendrimers synthesized by Roy and co-workers *(32)*.

The valency-corrected MIC does not tell the whole story *(4)*. For example, considering the polymer backbone (polyviologen) concentration at the MIC, the nearly fully threaded assembly **[21:21]** emerges as the best performer, with an MIC of 4.8 μM on a per-polymer basis. This value is half that of the Gal-1 concentration (10 μM in these experiments, and represents an advantage of 357-fold over lactose and 208-fold over **LCD** on a multivalent supramolecular complex to monovalent single-molecule basis. By considering the per-polymer concentrations necessary to obtain both the mixed and dispersed states, we uncovered a correlation *(4)* in efficacy with the number of connected lactoside ligands. This correlation is stronger for the dispersed state, and is independent of any other factors, such as the polyviologen chain length or the ligand spacing. For example, pairs such as **[4:8]**–**[5:21]** and **[8:8]**–**[10:21]** give similar results on a per-polymer basis for obtaining the dispersed state, in the absolute order **[4:8]** < **[5:21]** < **[8:8]** < **[10:21]**. A collorary to the simple dependence on *only* the number of connected ligands for the dispersed state is that other factors, such as the spacing of ligands and the lengths of the polymer chains they are located on, are correspondingly more important factors in describing the ability to attain the mixed state. We can equate the dispersed state with being at or near saturation on a hypothetical Gal-1 binding curve—since there is, by definition, not enough free Gal-1 available to aggregate the T-cells—and the mixed state with points lower on this hypothetical curve, closer to the IC_{50}. In this context, it is not too surprising that more subtle factors affect binding near a hypothetical IC_{50} than near saturation; alternatively, the mechanism(s) of binding may be changing with increasing pseudopolyrotaxane concentration on going from the mixed to dispersed states. A dependence on the number of connected ligands is

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a hallmark of the *stastical effect* in multivalent binding *(7-10, 37, 42)*. Here, the ligands are not connected directly by traditional covalent bonds, but are physically connected via the encircled polymer backbone. Hence, we observe a *supramolecular stastical effect* for these pseudopolyrotaxanes inhibiting Gal-1-mediated agglutination of the T-cells, particularly at the higher pseudopolyrotaxane concentrations that are necessary to obtain the dispersed state.

Quantitative Precipitation Assay

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We also studied *(4)* the ability of the pseudopolyrotaxanes to precipitate Gal-1 (**Figures 6-7**). It is well established that multivalent lectins and epitopes can induce each other to precipitate. If it is possible to distinguish between the lectin and carbohydrate portion of the precipitate, quantitative precipitations *(79)* can be performed to gain such information as the ratio of lectin to carbohydrate in the precipitate. The Bradford assay *(70-72)* was used as a protein-specific reporter, while UV spectroscopy provides the total amount of both protein and polymer in the solution and in the precipitate. At concentrations relevant to the precipitation assay, however, many of the assemblies and, in particular, the highly effective **[21:21]** and **[42:21]** do not yield a Bradford response above background, so that the assay can be used to report solely on protein concentration in the presence of pseudopolyrotaxane. Nevertheless, using this readout limits our ability to quantify precipitation results—unless there is no precipitation—to certain concentrations and degrees of threading, i.e., those that do not yield a significant Bradford response.

Neither the pseudopolyrotaxanes, their components, nor Gal-1 precipitate on their own, at least during the time-scale of these experiments. The individual pseudopolyrotaxane components—**LCD**, **PV-8**, and **PV-21**—do not induce Gal-1 to precipitate. When the pseudopolyrotaxanes are fully assembled, precipitation is rapid as measured by light scattering or by eye. A white precipitate is clearly visible within the first minute of mixing for concentrations such as 4:1 polyviologen repeating unit of assembly **[21:21]** to Gal-1 (50 μM). Based on light scattering experiments, all precipitations were complete at around 2 hours and so solutions were allowed to stand 3 hours before separating the precipitate and measuring the amount of Gal-1 and pseudopolyrotaxane in both the precipitate and the remaining solution.

The pseudopolyrotaxanes efficiently precipitate Gal-1 (**Figure 6A**), with assembly **[21:21]** inducing over 80% precipitation at a 4:1 starting ratio of the polyviologen repeating unit to Gal-1, and resulting in a 2:1 ratio of polyviologen repeating unit to Gal-1 in the precipitate. This ratio (**Figure 6B**) is relatively constant over a 10-fold titration range of assembly **[21:21]** to Gal-1, and represents a cross-linking Gal-1 occupying every other potential binding site in the precipitated pseudopolyrotaxane-lectin aggregates—an idealized model of this precipitate is shown in **Figure 7**. This ratio of polyviologen repeating unit

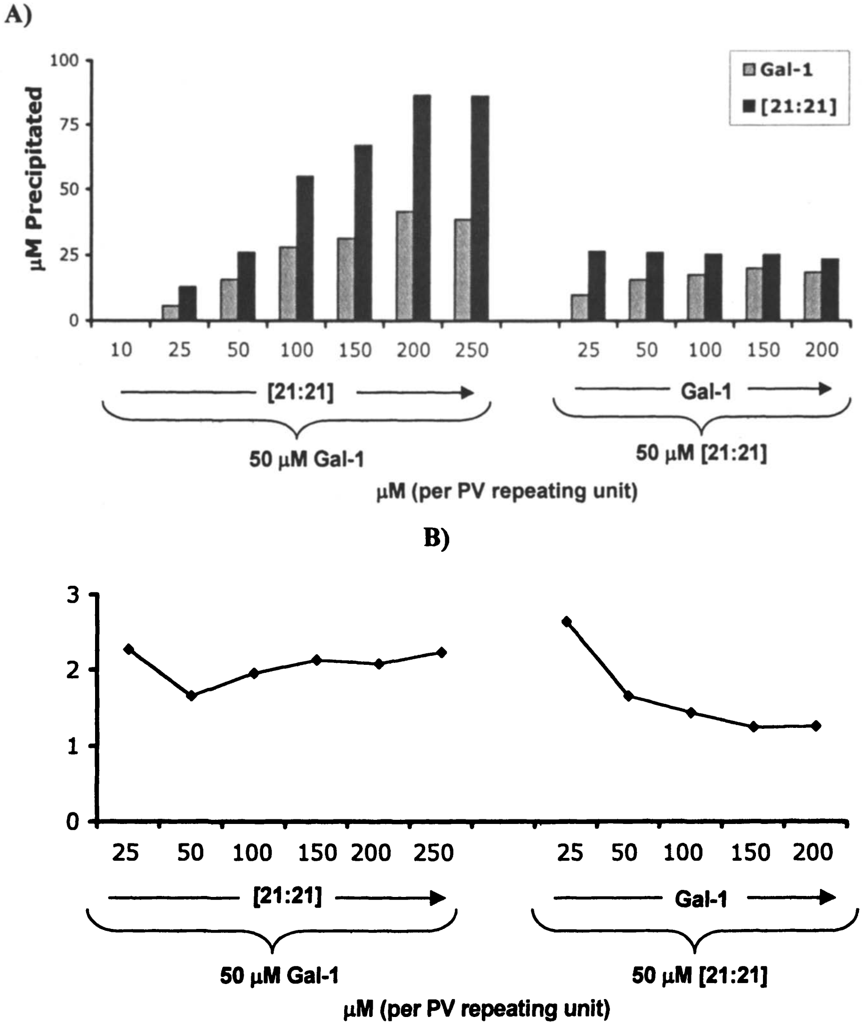

*Figure 6. A) Chart describing the concentrations of Gal-1 and pseudopolyrotaxane [21:21] in the precipitates obtained from two separate titrations. On the left, increasing concentrations of pseudopolyrotaxane [21:21] were mixed with a fixed concentration (50 µM) of Gal-1. On the right increasing concentrations of Gal-1 were mixed with a fixed concentration (50 µM) of pseudopolyrotaxane [21:21]. The concentrations of pseudopolyrotaxane [21:21] indicated are per polyviologen repeating unit. **B)** Chart comparing the ratio of Gal-1 and pseudopolyrotaxane [21:21] in the precipitates obtained from both titrations.*

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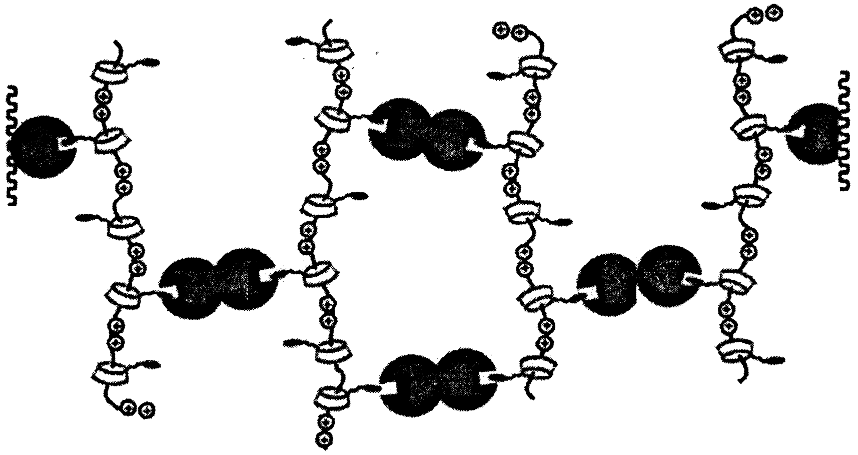

Figure 7. Idealized model of precipitate highlighting the 2:1 ratio of polyviologen repeating units to Gal-1 found in the precipitate across a 10-fold range of starting pseudopolyrotaxane [21:21] concentrations ***(Figure 6B).***

to Gal-1 in the precipitate can be driven to nearly 1:1 with an excess of Gal-1 relative to the polyviologen repeating units (**Figure 6B**). Note that this ratio is not expected to reach exactly 1:1, given that not every repeating unit of the polyviologen backbone will be occupied by an **LCD**. Normalized to the concentration of polyviologen repeating units, assemblies **[8:8]**, **[10:21]** and **[5:21]** were all less efficient at precipitating Gal-1 than assembly **[21:21]**, whereas assembly **[42:21]**, which contains an extra LCD for every polyviologen repeating unit, was somewhat more efficient than assembly **[21:21]**. This result was initially surprising, since free lactose, albeit at a huge excess, is used to solubilize the precipitate prior to analysis. Thus, free **LCDs** might have been expected to detract for precipitation efficiency. The precipitates from assembly **[42:21]** displayed Gal-1 to polyviologen repeating unit ratios close to 1:1, even at excess of polyviologen repeating unit to Gal-1 ratios. This observation reflects the increased likelihood of each polyviologen repeating unit to be threaded in assembly **[42:21]**, especially considering its resistance to dethreading (**Table 1**), and the propensity of pseudopolyrotaxane **[21:21]** to dethread over relatively short time-scale *(3, 4)*. This increased propensity to be threaded most likely accounts for the increased precipitation efficiency of pseudopolyrotaxane **[42:21]**, over-coming, or perhaps even being aided by, the equivalent of free **LCD**. That is, while a large excess of free lactoside clearly inhibits and reverses precipitation, one equivalent could aid precipitation by breaking up unproductive aggregates/chelates in favor of those more prone to precipitate.

Precipitation is occurring on a time-scale that is competitive with at least the beginnings of dethreading *(3, 4)*. Dethreading prior to exposure to Gal-1 can limit the effectiveness of precipitation, an effect which is noticeable relatively rapidly—as was observed when diluted solutions of assembly **[21:21]** were allowed to stand for 3 hours before beginning a precipitation experiment, which resulted in a small but noticeable decrease in the amount of Gal-1 precipitated—70% with a 4:1 ratio of polyviologen repeating unit to Gal-1 for **[21:21]**. Once exposed to Gal-1, cross-linking almost certainly alters the dethreading kinetics and thermodynamics.

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Outlook

Supramolecular species formed from over 20 (**[21:21]** and **[42:21]**) or as few as 3 (**[2:8]**) self-assembling components have been used successfully to inhibit Gal-1 in T-cell agglutination assays. These pseudopolyrotaxanes also rapidly and efficiently precipitate Gal-1. While other multivalent ligands tested displayed little to no enhancement in the valency-corrected MIC in the agglutination assay, improvements of 20-fold over **LCD** and 30-fold over lactose itself have been observed for the best-performing pseudopolyrotaxane (**[5:21]**). Considering the per-polymer MIC, pseudopolyrotaxane **[21:21]** is the best performer, with advantages of 357-fold over lactose and 208-fold over **LCD** on a multivalent supramolecular-complex to monovalent single-molecule basis. Pseudopolyrotaxane **[21:21]** is also able to precipitate over 80% of the Gal-1 from solution when the two are mixed at a molar ratio of 4:1 polyviologen repeating units to Gal-1.

There are many examples in biology *(80-82)* where individual proteins self-assemble into superstructures wherein the complexes gain or enhance functions with respect to those of the individual components. Although the time scale of assembly (3 – 30 days) is slower as a result of the viologen units on the polymer backbone, the pseudopolyrotaxanes are a rudimentary example of this phenomenon. Especially interesting in this context is the *supramolecular statistical effect* observed in the agglutination assay, where a dependence is observed for displayed lactosides connected to each other through mechanical and noncovalent interactions. Held in such a manner, the lactoside ligands are able to rotate freely and independently around the polymer backbone. The CDs can translate within the confines of the decamethylene units, which have a length that is twice the depth of a CD cavity. Not only do the lactoside ligands have the ability to "fine tune" their positions without enthalpic penalties having to be paid on account of inducing strain, but the unbound ligands may also reside away from the bulky protein in order to reduce steric clashes. The backbone itself, through its decamethylene segments, also lends considerable flexibility, especially compared to a chitosan backbone. The flexibility afforded by the polymer backbone, in conjunction with the dynamic property of its lactoside ligands, affords a supramolecular structure that mimics the fluidity and adaptability of cellular membranes, the biological target of Gal-1. Our results argue that flexible and adaptable ligands in general, and self-assembled multivalent pseudopolyrotaxanes in particular, show promise both for the study of protein-carbohydrate interactions and the exploitation of multivalency for targeting therapeutically relevant lectins.

Acknowledgements

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Chapter 20

De Novo Synthesis of Biofunctional Carbohydrate-Encapsulated Quantum Dots

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Semiconductor nanocrystals, also called quantum dots (QDs) are particles that exhibit unique size- and composition-dependent optical properties. Several recent reports have described the synthesis of QDs coated with a variety of biomolecules for cellular imaging applications. We have prepared QDs coated with the tumor-associated carbohydrate antigen (TACA) disaccharide Galβ1-3GalNAcα- (Thomsen Friedenreich antigen) O-conjugated to various linkers and studied their photoluminescent and binding properties. The *de novo* synthesis of highly stable, luminescent and functional quantum dots was achieved by the use of a "hybrid" system containing small percentages of different surface passivating agents along with an appropriate neoglycoconjugate. The glycan-coated quantum dots were prepared in a simple one-pot procedure that is amenable to various other saccharides. Initial studies with thiolacetic acid as a co-capping agent produced highly luminescent particles with functional carbohydrate units but the negative charge was undesirable for important applications such as cellular bioimaging. Replacement of this small organic acid with a short modified polyethylene glycol segment yielded quantum dots with very similar properties but lacking charged functionality on the surface. An array of binding and agglutination assays confirmed that the functional characteristics of the sugar were intact on all particles synthesized. These new tools hold great promise for labeling cells that express specific carbohydrate-binding proteins on their surfaces.

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Introduction

Exploring the preparation and properties of novel materials based on semiconductor nanocrystals or "quantum dots" (QDs) has evolved into a vibrant area of current research. QDs have received substantial attention due to their size-tunable optical properties and potential to develop into novel imaging agents.[1-8] QDs have the unique ability to absorb light within a broad range of wavelengths but emit photons in a very narrow band of the visible to near infrared spectrum. Recent advances in the synthesis of QDs of various sizes and compositions have bolstered the excitement for QD research, especially in the realm of biomedical applications (Figure 1).[9] Novel QDs encapsulated with biologically relevant receptors/ligands (peptides, proteins) have recently been prepared and shown to efficiently label specific cell types.[10-13] [14;15] [16;17]In addition, QDs that were injected into whole animals maintained bright luminescence after several weeks in vivo.[18] Near infrared-emitting QDs are now being employed as imaging agents in intact animals and a derivative of these has recently been shown to guide tumor surgery in mice.[19;20]

In contrast to proteins and antibodies, there have been few reports to date where carbohydrates have been linked to the surface of quantum dots.[16;17;21;22] Oligosaccharides represent a third class of biomacromolecules (along with proteins and nucleic acids) whose functions are broad and disease-relevant.[23] Cell surface carbohydrates that are covalently linked to proteins and lipids are involved in a myriad of important cellular functions that mediate adhesion, inflammation, tissue remodeling and metastasis. During tumor progression, the normal cellular carbohydrate repertoire is modified resulting in 1) a modification of physical properties and 2) the exposure of "non-self" structures called tumor-associated carbohydrate antigens (TACA) that elicit an immune response from the host. Thus, specific glycan-coated QDs have the potential to "label" cell types that display carbohydrate binding proteins (lectins) on their surface. Moreover, these particles could be used as multivalent displays of defined oligosaccharides. It could then be envisioned that these and other families of glyconanoparticles[24-26] have the potential to be used therapeutically as potent inhibitors of carbohydrate-protein interactions.[27;28] A recent report elegantly showed how sugar-coated QDs could be prepared through conjugation of

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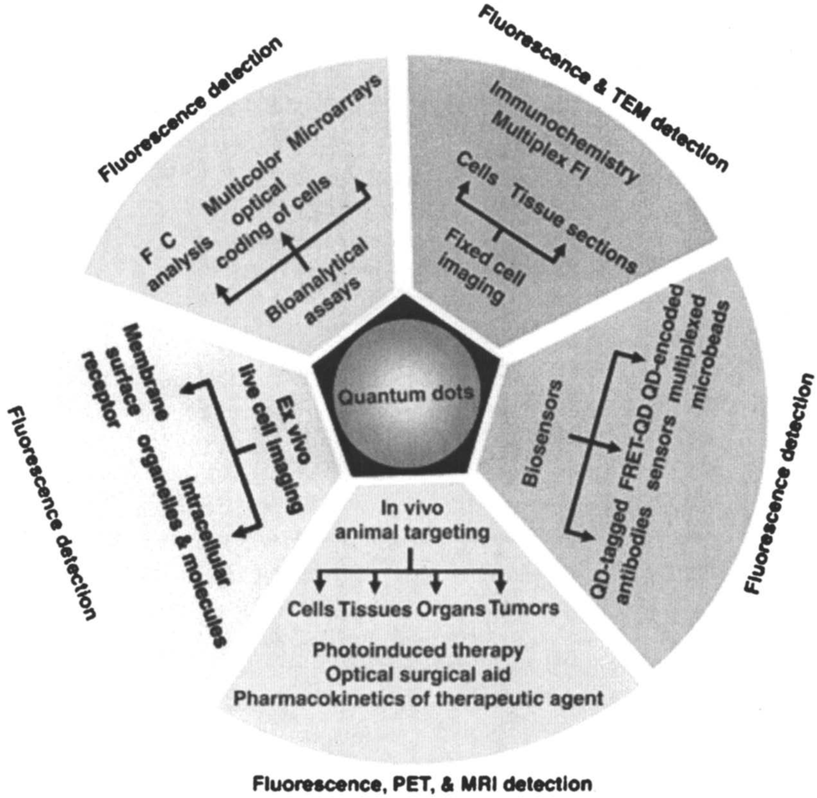
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Fluorescence detection
Biosensors
FRET-QD sensors
QD-encoded multiplexed microbeads
QD-tagged antibodies
Quantum dots
In vivo animal targeting
Cells Tissues Organs Tumors
Photoinduced therapy
Optical surgical aid
Pharmacokinetics of therapeutic agent
Fluorescence, PET, & MRI detection
Fluorescence detection
Membrane surface receptor
Ex vivo live cell imaging
Intracellular organelles & molecules

Figure 1. Applications of quantum dots in biological imaging. Reprinted with permission from Science *2005,* 307 *(issue 5709, 28 Jan), 538-544; copyright AAAS, all rights reserved). (See page 2 of color inserts.)*

appropriately functionalized saccharides to other molecular scaffolds previously conjugated to the QD surface.[16]

Our laboratory is interested in discovering novel scaffolds for the multivalent presentation of TACA's, in particular the Thomsen-Freidenreich antigen (T$_f$-ag), a disaccharide (Galβ1-3GalNAc) readily detectable in *ca.* 90% of all primary human carcinomas and their metastases but rarely in normal tissues.[29;30] T$_f$ antigen also plays a leading role in docking breast and prostate cancer cells onto endothelium by specifically interacting with endothelium-expressed galectin-3.[31;32] and the presence of T$_f$-ag activates endothelial cells to overexpress this lectin.[33] Thus, a multivalent QD sugar template is very attractive since it could serve the dual role of acting as both an inhibitor of tumor cell attachment and metastasis and/or a label for cells expressing specific lectins. Herein we report the synthesis of highly stable QDs that are coated with directly attached saccharides and provide several results confirming the functionality of the attached saccharides.

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Results and Discussion

Many synthetic procedures have been developed for QD synthesis, of which most all use an organic soluble complexing agent to stabilize the nanocrystals by surface passivation. The method of Bawendi and colleagues is considered the "classic" procedure for the preparation of highly crystalline and uniform QD's.[34] This method is based on the high temperature thermolysis of metallic precursors employing trioctylphosphine oxide (TOPO) as a surface passivating agent. The resulting hydrophobic QDs are rendered water-soluble through exchange of TOPO molecules with hydrophilic thiols[13;35;36] that serve to displace the TOPO molecules due to the highly thiophilic nature of the QD core/shell metals (e.g., CdSe, CdTe, ZnS, CdS). For example, mercaptoacetic acid (MAA) was originally used for solubilizing TOPO-coated QDs.[13] Subsequently, the MAA can be displaced with other hydrophilic ligands or conjugated with biomolecules through standard (peptide) coupling chemistry. In order to prepare an appropriate linked Tf-ag unit as a precursor for QD synthesis, the olefin of **1**[37] (Scheme 1) was readily transformed into thiol **3** via thioacetate **2** by radical addition of thiolacetic acid followed by acid-catalyzed debenzylidenation and Zemplen removal of the acetates in very high overall yield as described for our synthesis of T_f-ag-coated gold nanoparticles.[38] Thiol **3** dimerizes on storage to the *bis*-T_f disulfide that could be cleaved by dithiothreitol (or other reducing agents) prior to use in QD synthesis.

Initially, we attempted the displacement of the surface-capping TOPO layer with the water- and methanol- soluble thiol **3** using commercially available CdSe/ZnS core-shell QDs (Evident Technologies, http://www.evidenttech.com). All our efforts led to either incomplete displacement or to *very fragile QDs* which did not survive mild ultrafiltration or dialysis; the particles flocculated (formed fluffy aggregates) soony after the free ligand was removed from the solution.

At this point, we turned our attention to an aqueous synthesis reported recently to produce highly luminescent thiol-capped CdTe/CdS core-shell nanocrystals.[39] This study had shown that the use of MAA as a capping agent accelerated the growth of CdTe QDs while maintaining moderate luminescence. When the synthesis was carried out with thiol **3** alone, nanocrystals obtained over 48 hours had very low green luminescence. When ca. 3:1 ratio of thiol **3** to

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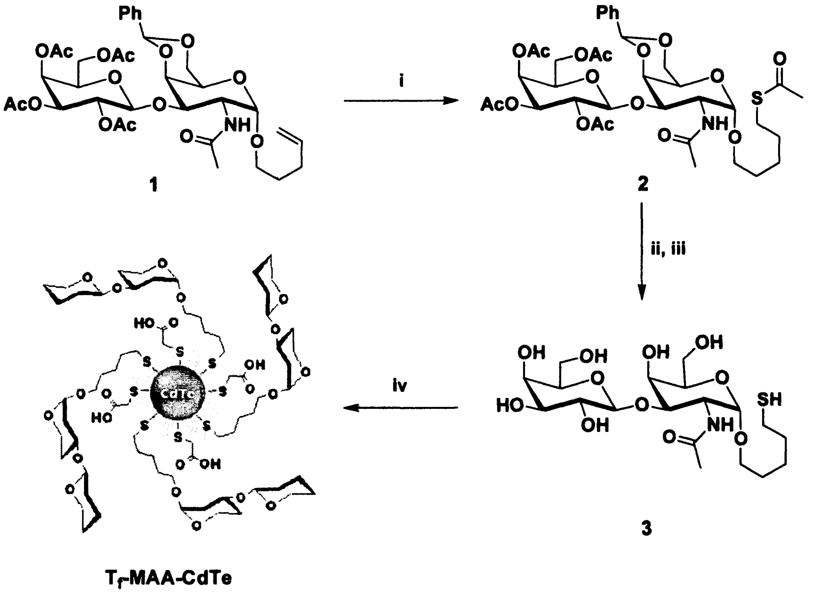

Scheme 1. (i) HSAc/AIBN/1,4-dioxane/70 °C, quant.; (ii) 80% AcOH, 60 °C, 70%; (iii) NaOMe/MeOH, 98%; (iv) $HSCH_2CO_2H$, $Cd(ClO_4)_2$, NaHTe, H_2O/100 °C (See page 3 of color inserts.)

MAA was employed, under identical conditions the crystal growth proceeded much more rapidly and bright yellow luminescence was observed after only 39 hours (Figure 2). Thus synthesized hybrid T_f-MAA-CdTe QDs were purified by ultrafiltration and after lyophilization yielded stable nanocrystals that were freely soluble in water and DMSO. The measured quantum efficiency (QE) of the T_f-MAA-CdTe QDs (10%, see experimental) was comparable to hydrophilic QDs coated solely with MAA.[39] These nanocrystals were remarkably stable since solutions of pure QDs in water stored in the dark at 4 °C for at least 6 months showed no signs of decreased luminescence, flocculation, or ligand leaching. Interestingly, similarly prepared MAA, mercaptoproprionic acid (MPA) and thioglycerol-capped QDs would flocculate in a few days when stored in aqueous solution in the absence of free ligand. To our knowledge, *this represents the first one pot de novo synthesis of carbohydrate functionalized quantum dots.*

Several lines of evidence showed that the chemical composition and functional integrity of the disaccharide unit remained intact after attachment to the QD. Figure 3 shows the ^{1}H NMR spectra of the free thiol 3 and the hybrid T_f-MAA-CdTe QDs in D_2O. The broad peaks in the spectrum of the QDs are a result of increased relaxation rates due to effective molecular weight (>50 KDa)

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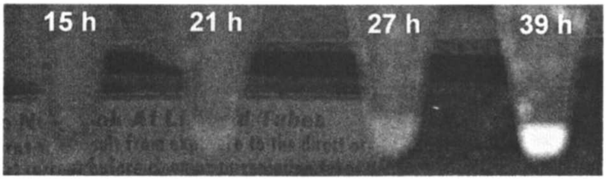

Figure 2. Luminescence increase and color change of the hybrid T_f-A/MAA CdTe QDs depending on the duration of reflux. (See page 2 of color inserts.)

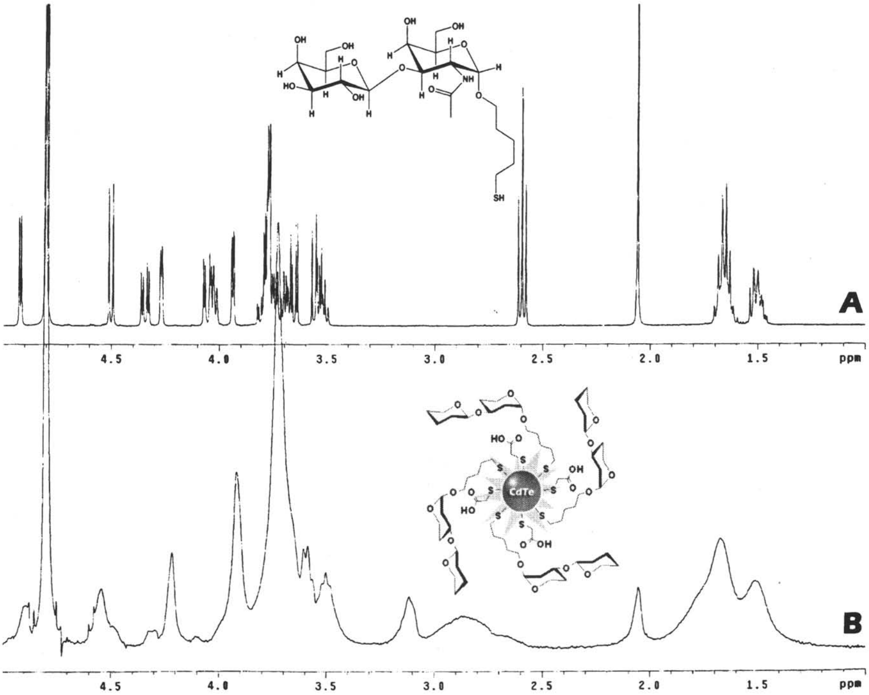

Figure 3. NMR spectra in D_2O of (a) Thiol 3; (b) T_f-MAA-CdTe QDs. (See page 4 of color inserts.)

of the nanoparticles and the close packing of the ligands on their surface. The proton chemical shifts of the QDs confirmed that the capping T_f molecules have retained the structure of the free thiol.

Figure 4 shows binding between a T_f –specific monoclonal antibody[40] (mAb) and T_f-MAA-CdTe QDs as detected by laser scanning confocal microscopy. A clear, time-dependent aggregation of the QDs is evident after addition of the mAb. Panels (C) and (D) show a control QD prepared with thioglycerol alone (to partially mimic the hydroxyl-coated sugars) with no visible aggregation after 1 hour post mAb treatment. Similar results were obtained by incubation with peanut agglutinin (PNA) and with detection by transmission electron microscopy (TEM). Large clusters of agglutinated QDs were readily observed and measurements of the size of the aggregates could be made (data not shown). These results confirm that the functional integrity of the antigen is preserved while attached to the QD.

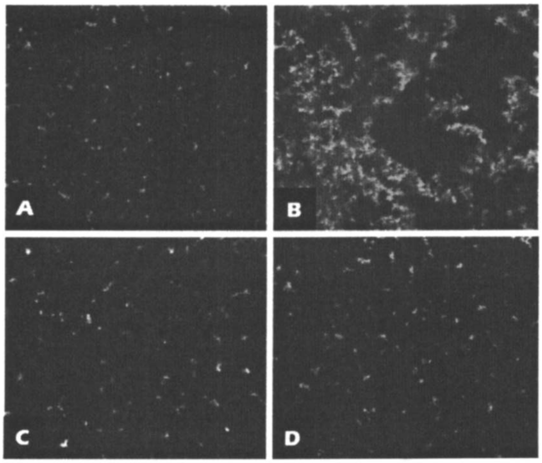

Figure 4. Confocal micrograph images of T_f-MAA-coated QDs (a) alone (b) 5 min after addition of anti-T_f mAb[12] Panels (c) and (d) show images of a thioglycerol-coated QD alone and 45 min after addition of antibody, respectively. (See page 4 of color inserts.)

Other independent proof that T_f antigen conjugated to QDs retained its biofunctional properties was obtained from Lectin Affinity Chromatography (LAC). Thus, agarose-bound mannose-binding *Pisum sativum* agglutinin (PSA) and T_f ag-specific peanut agglutinin (PNA) were loaded with equal amounts of T_f-MAA-CdTe QDs and eluted with PBS buffer. The QDs eluted readily from the PSA column but were strongly retained on the PNA column. The T_f-MAA-CdTe QDs were eluted from the PNA column with 200 mM solution of

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competing D-galactose in PBS, confirming the recognition of the glycan antigen on the T_f-MAA-CdTe QDs.

Polyethyleneglycol (PEG) Modifications/Removal of Charge from Hybrid QDs

The influence of the carboxyl functionality on the photoluminescene of mercaptocarboxylic acid stabilized CdTe nanocrystals has been recently investigated by Gao et al.[41] The study suggested that the acids strongly interacted with the nanocrystals via coordination between carboxyl groups and cadmium ions on the particle surface. This advantage notwithstanding, for biological applications the presence of charged sites on the nanoparticle surface may interfere with ligand recognition and increase non-specific binding interactions to sites of opposite charge. Thus it would be extremely useful to discover other neutral functional groups that could be used as efficient stabilizers of cadmium deficiency sites on the surface of QDs. In one report, highly luminescent CdS nanoclusters were prepared by arrested precipitation in polyethylene glycol (PEG-200) as a solvent.[42] It has been proposed that PEG may provide a favorable environment for suspending high-luminescence yield CdS nanoclusters via its ability to strongly bind positive ions, much like the crown-ethers. To test this hypothesis we first prepared CdTe QDs stabilized by commercially available methoxy(polyethylene glycol) thiol with a molecular weight of 2 KDa (mPEG2000-SH, www.sunbio.com). This preparation afforded highly luminescent water soluble mPEG2000-CdTe QDs. Since the mPEG2000 chain was significantly longer than the T_f linker and would potentially hinder sugar recognition in a hybrid T_f-mPEG2000 particle, we synthesized a short mercapto derivative of methoxy(triethylene glycol) (mTEG-SH) and conjugated it to the CdTe QDs by a similar procedure as described above (Scheme 2). The resulting quantum dots were also highly luminescent with a quantum yield similar to MAA-stabilized CdTe QDs. Since these QDs displayed acceptable luminescence, we carried out the synthesis of hybrid T_f-mTEG-CdTe QDs similar to the preparation of T_f-MAA-CdTe QDs above with a ratio of 3:mTEG-SH = 3:1 (Scheme 2).

This gave highly luminescent QDs (QE = 10%) with a fluorescence width at half maximum (FWHM) of 38 nm, which is comparable to commercially available QDs (FWMH= 30-40 nm, see www.qdots.com for commercial data). Figure 5 shows the NMR spectra of mTEG-SH alone and the hybrid T_f-mTEG-CdTe QDs in D_2O. It is evident that both the T_f antigen and the mTEG have been incorporated into the QDs shell. The functional properties of the T_f-mTEG-CdTe QDs were very similar to those of the T_f-MAA-CdTe QDs (mAb-induced agglutination, behavior in LAC assays) and hence we succeeded in preparing a neutral "surrogate" for our first generation negatively charged QDs. Preliminary cell binding experiments have shown that the substitution of mTEG for MAA in the hybrid QDs completely removes non-specific interactions that plagued the charged T_f-MAA-CdTe QDs (data to be published elsewhere). This useful property of PEG incorporation for maintenance of water solubility while removing protein binding

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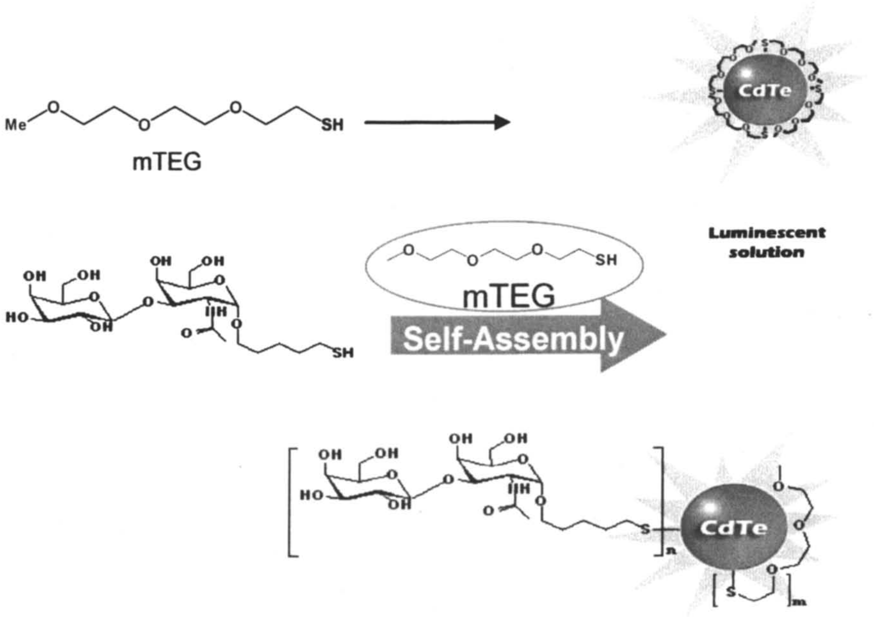

Scheme 2.
(See page 3 of color inserts.)

has been exploited on numerous occasions including reports regarding their use in surface coating of both gold nanoparticles [43] and quantum dots. [12]

In addition to adding PEG as a "co-solvent" in the preparations of QDs, PEG chains could also be attached to the anomeric center of the disaccharide as ligands for the preparation of other novel sugar-coated QDs. In this vein, we employed the same hexaPEG linker we used previously for the preparation of T_f ag-coated gold particles.[38] The hybrid construction of the Tf disaccharide linked via a hexaPEG thiol with 25% of mTEG yielded a robust and stable QD (Figure 6) that is being used for various biolabeling experiments.

In summary, a simple, one-pot aqueous synthesis of robust, luminescent T_f-MAA(mTEG) hybrid CdTe quantum dots is reported for the first time. The novelty of this method is several-fold. First is the use of two distinct capping agents, one that promotes the growth and luminescence and the other that provides biofunctionality. Second, the synthesis can be carried out under relatively low temperature and under different pH conditions with no detrimental effect on the carbohydrate unit. Third, the color (size) of the QDs is controlled solely by duration of the synthesis. Fourth, these QDs maintain their stability in the absence of free ligand in solution. With the incorporation of mTEG into

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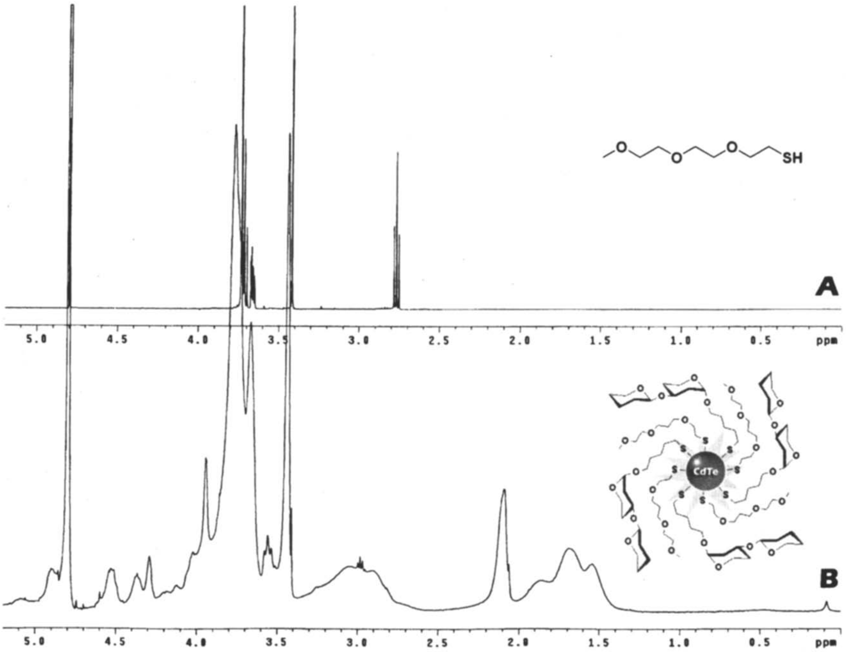

Figure 5. NMR spectra in D_2O of (a) free mercapto mTEG (mTEGSH) and (b) T_f-mTEG-CdTe hybrid QDs. (See page 5 of color inserts.)

hybrid nanocrystals, there is now available a useful model system for further development of tumor stage-specific, biocompatible imaging agents.

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Experimental

Melting points were determined on Fisher-Johns melting point apparatus and are uncorrected. R_f values refer to TLC performed on Analtech Uniplates GF pre-coated with silica gel 60 to a thickness of 0.25 mm. The spots were

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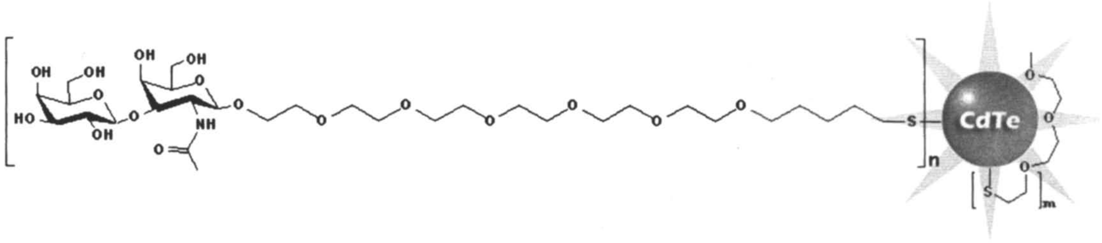

Figure 6. Structure of the optimum combination of linker and co-passivating agent for a biofunctional and robust T$_f$-coated QD.
(See page 5 of color inserts.)

visualized by charring with a solution of ammonium molybdate (IV) tetrahydrate (12.5 g) and cerium (IV) sulfate tetrahydrate (5.0 g) in 10% aqueous H_2SO_4 (500 mL). Flash column chromatography (FCC) was performed under medium pressure using silica gel 60 (230-400 mesh, E. Merck) or C18 reverse phase (RPFC) silica and usually employed a stepwise solvent polarity gradient, correlated with TLC mobility.

NMR spectra were recorded on Varian InovaUnity-400 instrument with residual $CHCl_3$ (7.26 ppm) or HDO (4.80 ppm) as the internal standard at frequencies of 399.74 MHz for ^1H and 100.51 MHz for ^{13}C. IR spectra were taken with JASCO FT/IR-615 spectrometer. Specific optical rotations were determined using JASCO-P1010 polarimeter in 0.5 dm cuvette at 589 nm in chloroform. Five consecutive measurements were taken each time; the average value is given. Positive ion fast-atom bombardment mass spectra (FABMS) were obtained at an accelerating voltage of 6 kV and a resolution of 2000. Glycerol was used as the sample matrix, and ionization was effected by xenon atoms. Elemental analyses were performed by Atlantic Microlab, Inc., Norcross, GA. Laser scanning confocal microscopy was performed on Zeiss 510 confocal microscope (NCI-Frederick, Confocal Microscopy Facility, Image Analysis Lab).

Dialysis Slide-A-Lyzer® cassettes (MWCO 10KDa) were from Pierce (www.piercenet.com). Centriplus® centrifugal filter devices (MWCO 10KDa, 30KDa, 50 KDa, 100KDa) were from Millipore (www.millipore.com). TOPO coated core-shell CdSe/ZnS nanocrystals (quantum dots, QDs) were purchased from Evident Technologies (www.evidenttech.com). The following nanocrystals were used in this work: blue (d=3.7 nm, λ_{em}=490 nm), green (d=4.3 nm, λ_{em}=520 nm), and red (d=7.3 nm, λ_{em}=620 nm). Unless otherwise noted, all other materials were purchased from Aldrich-Sigma (www.aldrich.com) and used without further purification. Al_2Te_3 was purchased from Cerac, Inc. (www.cerac.com).

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Preparation of T_f-Encapsulated CdSe/ZnS Nanocrystals via TOPO Ligand Displacement.

Method A: Phase-Transfer from Organic to Aqueous Phase

A solution of thiol 3 (20 mg, 0.041 mmols) in 2 ml of UltraPue® water was adjusted to pH 10 with concentrated teramethylammonium hydroxide pentahydrate (TMAH) in water. This solution was added to 1 ml of TOPO-coated CdSe/ZnS QDs (Evident Technologies) in toluene (0.5 mg/ml) and reaction vessel was sealed under argon. The reaction temperature was adjusted to 60 °C for 2 hours and then the reaction was left overnight at ambient temperature with vigorous stirring. The organic phase became colorless with no luminescence while the aqueous layer had the color and luminescence of the nanocrystals. The aqueous phase was isolated, washed 3x with Et_2O, diluted with water, concentrated by ultrafiltration on Millipore Centriplus® YM-30 (MWCO 30KDa) to 100μl and freeze-dried. Dry QDs were freely soluble in water but flocculated after only a few days in solution at 4 °C. Phase transfer from Et_2O or from $CHCl_3$ produced similar results. NMR of thus prepared QDs shows incomplete (ca. 1:1) displacement of TOPO. Noteworthy are the two signals appearing around 0 ppm. These signals presumably correspond to the CH_2-S-methylenes of the sugar (0 ppm) and methylenes of the octyl chain (-0.1 ppm) of TOPO [$(CH_2)_3P=O$] next to the QD surface. The signal at –0.1 ppm disappeared when TOPO was completely displaced by the sugar. This upfield signal was also observed in chloroform solution of the commercial TOPO-CdSe/ZnS QDs.

Method B: Direct TOPO Dispacement in Methanolic Suspension

A solution of 20 mg (0.041 mmols) of thiol 3 in 1 ml MeOH was basified to pH 10 by dropwise addition of concentrated TMAH in MeOH. This solution was then transferred to the stirred suspension of 0.5 mg of TOPO-coated CdSe/ZnS QDs in 1 ml of MeOH *via* syringe under argon. The combined solution was purged with argon for 20 min and the vessel was sealed. The solution was stirred for 24 hours at 50 °C. The suspension was centrifuged to give a colored precipitate. TLC of the supernatant showed that excess thiol remained in solution when the reaction was stopped. The precipitate was washed with MeOH (4x5ml), centrifuged and decanted. Vacuum-dried QDs were soluble in water. NMR of the QDs in D_2O showed complete displacement of TOPO molecules with TMAH being the major impurity. Attempted purification of the QDs by dialysis on Pierce's Slide-A-Lyzer® Dialysis Cassette (MWCO 10KDa) or ultrafiltration on Centriplus® YM-50 (MWCO 50KDa) resulted in complete flocculation of the QDs. Flocculated particles were no longer soluble in water.

Preparation of T_f-CdTe QDs via Self-Assembly in Aqueous Solution

A solution of 50mM NaHTe was prepared by passing H_2Te gas (generated by reaction of 123mg (0.28 mmols, 0.85mmols in Te) of Al_2Te_3 with 10 ml of 0.5M H_2SO_4) with a slow flow of argon through 10 ml of de-aerated 50mM NaOH solution. The resulting NaHTe solution was light purple and clear. Separately, a solution of 28 mg (0.058 mmols) of thiol 3 in 700 μl (0.011 mmols) of 16mM $Cd(ClO_4)_2·H_2O$ was prepared, adjusted to pH 10 with 1M NaOH and purged with argon for 20 min. To this solution was added 115 μl (0.0057 mmols) of the freshly prepared solution of NaHTe very rapidly by syringe. The reaction solution immediately turned light-orange. The final ratio of reagents was as follows: $Cd^{2+}:Te^{2-}:3$ = 1:0.5:5.2. The reaction was set to reflux under open air. In a few minutes, the solution became yellow. During the reflux 50 μl aliquots were collected and analyzed for UV absorption. Rapid growth

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during this time is evident from the shift of the absorption maxima to longer wavelengths.[5] After 48 hours only faint green luminescence was observed. The solution was cooled to ambient temperature, diluted with water and purified from low MW impurities on Centriplus® YD-30 (MWCO 30KDa) centrifugal device. Lyophilization of the purified solution gave 5 mg of T_f-CdTe QDs as a pale yellow fluffy substance which was freely soluble in water. Absence of sharp peaks in the ^1H NMR spectrum indicates that no free ligands are present in solution.

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Preparation of Hybrid T_f-MAA-CdTe QDs via Self-Assembly in Aqueous Solution

A solution of 12.3 mg (0.025 mmols) of thiol 3 and 6 μl (8 mg, 0.085 mmols) of mercaptoacetic acid in 1400 μl (0.022mmols) of 16mM solution of $Cd(ClO_4)_2 \cdot H_2O$ was adjusted to pH 10 with 1M NaOH and the solution was purged with argon for 20 min. At this time, 230 μl (0.011 mmols) of a freshly prepared 50mM NaHTe solution (see above) was rapidly added by syringe under argon. The reaction solution immediately became light-orange. The ratio of reagents was as follows: Cd^{2+}:Te^{2-}:RSH [thiol 3:MAA] = 1:0.5:5 [1:3.4]. The reaction was set to reflux under open air. In a few minutes solution became yellow. During the reflux 50 μl aliquots were collected in 15h, 21h, 27h and 39h and analyzed for UV absorption and luminescence. In 39 hours bright yellow luminescence was observed. The solution was cooled to the ambient temperature, diluted with water and purified from low MW impurities on Centriplus® YD-50 (MWCO 50KDa) centrifugal device. Lyophilization of the purified solution gave 4 mg of T_f-MAA-CdTe hybrid QDs as orange fluffy substance which was freely soluble in water. NMR of the T_f-MAA-CdTe solution in D_2O showed that all peaks from the starting disaccharide were evident but broad due to the size and electronic nature of the nanocrystals.

Preparation of mPEG2000-CdTe QDs via Self-Assembly in Aqueous Solution

A solution of 500 mg (0.25 mmols) of mPEG2000-SH (www.sunbio.com) in 8 ml of 16 μM $Cd(ClO_4)_2$ was purged with argon for 20 minutes (the initial pH of the solution was 3.04; no pH adjustment was made). A solution of freshly prepared 50mM NaHTe (1.33 ml, 0.066 mmols) was briskly injected under argon. The reaction solution immediately became yellow and was heated to reflux under open air. Development of luminescence was followed by withdrawing 50μl aliquots at regular intervals. A weak greenish luminescence was observed after 19 hours of reflux. After 42 hours, the reaction solution was red-orange and showed strong orange luminescence under UV light (λ_{max}= 350 nm). The solution was cooled and purified by ultrafiltratin on Centriplus-YD50 (MWCO 50KDa), freeze-dried, redissolved in D_2O and analyzed by ^1H and ^{13}C NMR as for the nanocrystals prepared above.

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Preparation of mTEG-CdTe QDs via Self-Assembly in Aqueous Solution

A solution of 120 mg (0.667 mmols) of mTEGSH in 21 ml of 16μM $Cd(ClO_4)_2$ was purged with argon for 10 minutes (initial pH of the solution was 2.52; no pH adjustment was made). A solution of 50mM freshly-prepared of NaHTe (3.5 ml, 0.175 mmols) was added swiftly under argon. Reaction solution immediately became yellow-orange. The ratio of reagents was as follows: 3.8 TEGSH: 2 Cd^{2+}: 1 Te^{2-}. When heating to 100 °C began, a greenish-yellow precipitate formed on the flask wall. Cooling of the solution to room temperature resulted in complete dissolution of the precipitate. The reaction solution was refluxed overnight. In 16 hours the color of the precipitate changed from green to orange while the reaction solution remained clear and nearly colorless. The pH of the solution was 3.6. When the reaction was cooled to ambient temperature orange precipitate completely dissolved and the resulting orange solution had bright green luminescence under UV light (λ_{max}= 350nm). The solution of mTEG-CdTe quantum dots was purified on 50KDa MWCO, lyophilized, redissolved in D_2O and analyzed by NMR. Remarkably, the solution of thus prepared mTEG-CdTe QDs readily formed orange precipitate/suspension on heating to 60-70 °C but redissolved readily upon cooling to RT. The heating-cooling cycle could be repeated indefinitely without any noticeable deterioration of the nanocrystals.

Preparation of Hybrid T_f-mTEG-CdTe QDs via Self-Assembly in Aqueous Solution

A solution of 26 mg (0.0054 mmols) of 3 and 28 mg (0.16 mmols) of mTEGSH in 5 ml of 16μM $Cd(ClO_4)_2$ (0.09 mmols) was purged with argon for 10 minutes (initial pH 2.9; no pH adjustment was made). To this solution was quickly added 875 μl of freshly-prepared 50 mM NaHTe. The reaction solution immediately turned brown. The ratio of reagents was 4.9 RSH (1x TFSH: 3x TEGSH) : 2 Cd^{2+} : 1 Te^{2-}. When heating to reflux commenced in the open air, the color of the solution changed to bright-yellow. After 42 hours the reaction was cooled and purified on Centriplus ultrafiltration filter with MWCO 50KDa. The luminescence of the mixed TF-TEG-CdTe quantum dots was brilliant green. The solution was lyophilized, redissolved in D_2O and analyzed by NMR.

Quantum Yield Determination

Quantum efficiency was determined on a FluroMax-2 spectrofluorimeter (Jobin Yvon, www.jyhoriba.co.uk) using an excitation wavelength of 490 nm. Spectra were recorded in Starna® 2x10mm quartz cells. Since the photo-physical properties of fluorescein most closely resembled those of the T_f-encapsulated quantum dots (T_f-MAA-CdTe), we chose this dye as our standard. A solution of fluorescein in 0.1 M NaOH has an absorption maximum at λ_{max} = 490 nm and an emission maximum at λ_{max}=513 nm while the quantum dots have a first absorption maximum at $\lambda = 460$ nm and emit at $\lambda_{max} = 520$ nm. Six dilutions each of fluorescein and the T_f-MAA-CdTe QDs were prepared with $A_{490} = 0.000$, 0.021, 0.033, 0.055, 0.075, 0.100 and $A_{490} = 0.000$, 0.015, 0.023, 0.040, 0.064, 0.087, respectively. Integrated fluorescence intensity was calculated from the fully corrected fluorescence spectra.

Lectin Affinity Chromatography (LAC)

Agarose-bound galactose-specific peanut agglutinin (PNA) and mannose/glucose-specific *Pisum sativum* agglutinin (PSA) were purchased from Vector Labs, Burlingame, CA (www.vectorlabs.com). Equal amounts (1 mL) of agarose-immobilized PSA and PNA were loaded onto two separate columns and washed with 10 bed volumes of 1x PBS at pH 7.4 to wash out the lectin-stabilizing sugars. Solutions of T_f-MAA-CdTe QDs in water (50 µl) were loaded onto each column and further soaked in with 150 µl of 1x PBS. After 10 minutes each column was washed with 10 bed volumes of 1x PBS in 1ml aliquots. Each aliquot was compared to the solution of 50µl T_f-MAA-CdTe QDs in 1ml 1x PBS by UV-Vis spectroscopy. No QDs were eluted from the PNA column in any of the 10x1ml aliquots while elution of QDs from the PSA column was complete in the first 1ml aliquot. Further, washing of the PNA column with several 1ml aliquots of 200 mM galactose displaced the QDs bound to the PNA thus indicating reversibility of the binding. UV-Vis spectra showed that the QDs were completely displaced from the column in the first three aliquots of the galactose elutions.

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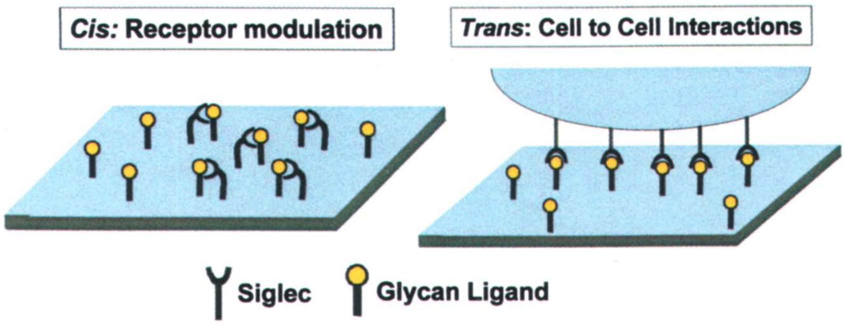
Cis: Receptor modulation
Trans: Cell to Cell Interactions
Siglec
Glycan Ligand

Figure 1.1. CD22 can interact with ligands both cis and trans. (Adapted with permission from reference 20. Copyright 2004 Elsevier.)

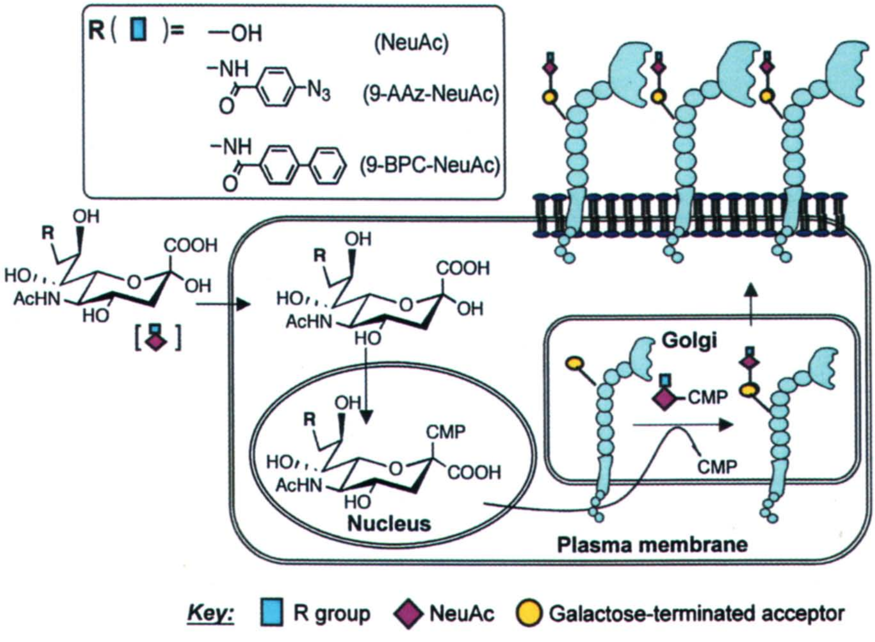
R ( ) =
—OH
(NeuAc)
–NH
O
N3
(9-AAz-NeuAc)
–NH
O
(9-BPC-NeuAc)
OH
R
COOH
HO
AcHN
O
OH
HO
COOH
CMP
Nucleus
Golgi
CMP
CMP
Plasma membrane
Key:
R group
NeuAc
Galactose-terminated acceptor

Figure 1.2. Strategy for introducing 9-substituted NeuAc into cell surface glycoprotein ligands of CD22. Modified sialic acids can be taken up by cells, converted to CMP-sialic acid, and transferred to nascent glycoproteins by sialyltransferases in the Golgi apparatus, and transported to cell surface. (Adapted with permission from reference 25. Copyright 2005 Nature Publishing.)

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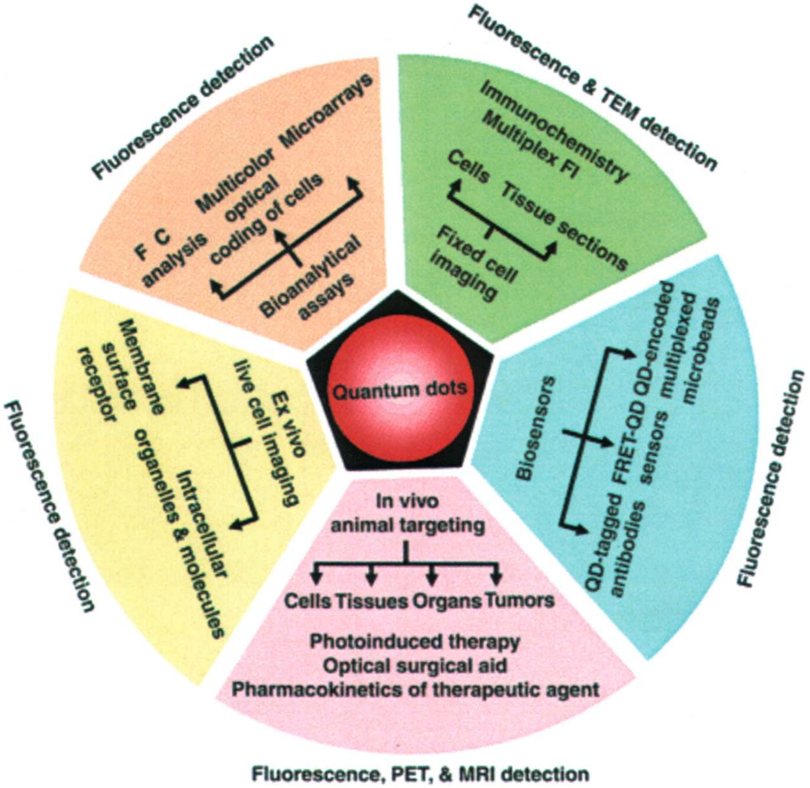
Fluorescence detection
F C analysis
Multicolor optical coding of cells
Microarrays
Bioanalytical assays
Fluorescence & TEM detection
Immunochemistry
Multiplex FI
Cells
Tissue sections
Fixed cell imaging
Fluorescence detection
Biosensors
FRET-QD sensors
QD-encoded multiplexed microbeads
QD-tagged antibodies
Fluorescence, PET, & MRI detection
In vivo animal targeting
Cells
Tissues
Organs
Tumors
Photoinduced therapy
Optical surgical aid
Pharmacokinetics of therapeutic agent
Fluorescence detection
Ex vivo live cell imaging
Membrane surface receptor
Intracellular organelles & molecules
Quantum dots

Figure 20.1. Applications of quantum dots in biological imaging. Reprinted with permission from Science *2005,* 307 *(issue 5709, 28 Jan), 538-544; copyright AAAS, all rights reserved).*

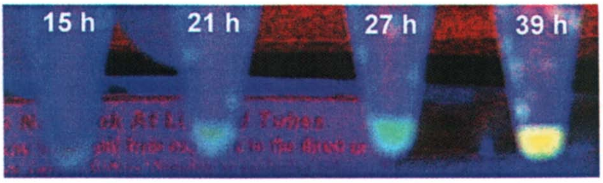
15 h
21 h
27 h
39 h

Figure 20.2. Luminescence increase and color change of the hybrid T_f-A/MAA CdTe QDs depending on the duration of reflux.

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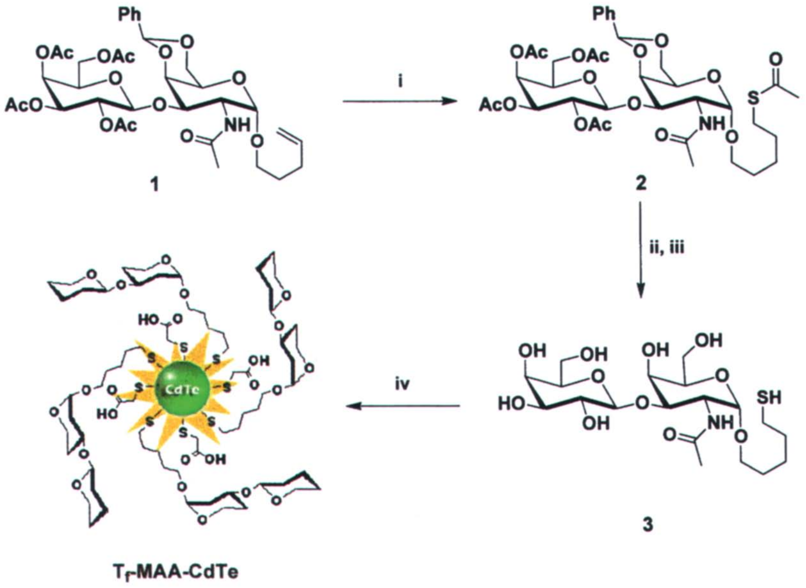
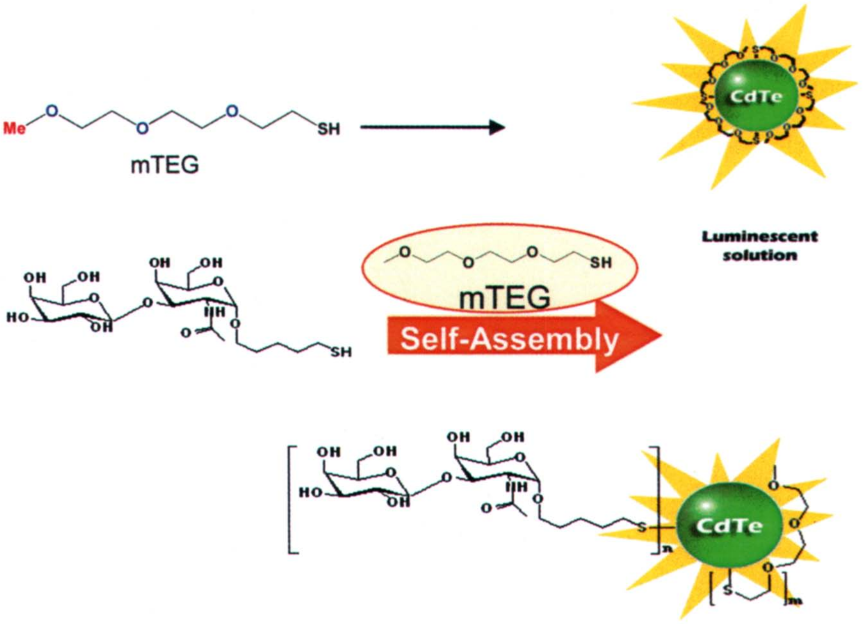

1
i
2
ii, iii
3
iv
CdTe
T_f-MAA-CdTe

Scheme 20.1. (i) HSAc/AIBN/1,4-dioxane/70 °C, quant.; (ii) 80% AcOH, 60 °C, 70%; (iii) NaOMe/MeOH, 98%; (iv) $HSCH_2CO_2H$, $Cd(ClO_4)_2$, NaHTe, H_2O/100 °C


mTEG
CdTe
Luminescent solution
mTEG
Self-Assembly
CdTe

Scheme 20.2

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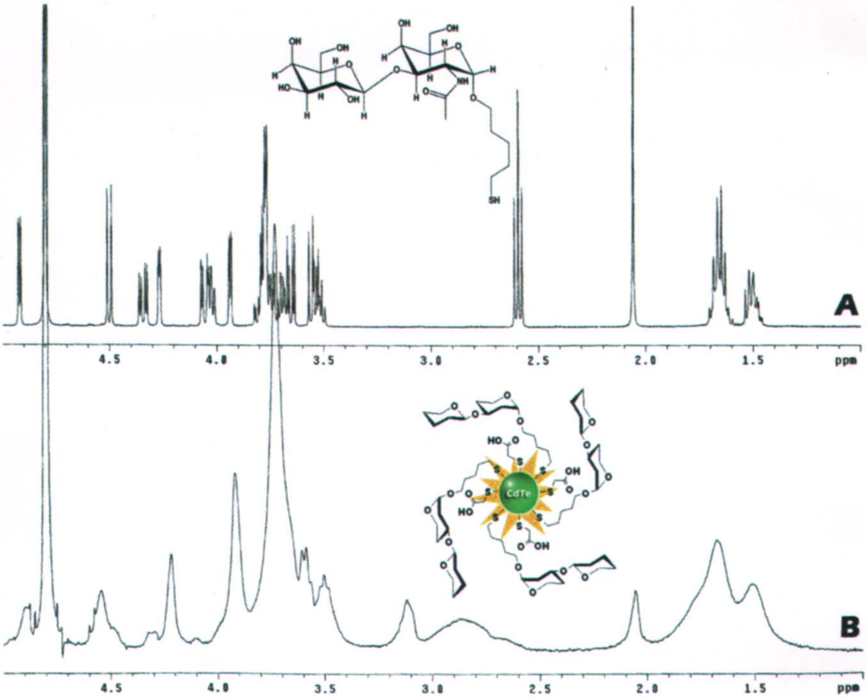

Figure 20.3. NMR spectra in D_2O of (a) Thiol 3; (b) T_f-MAA-CdTe QDs.

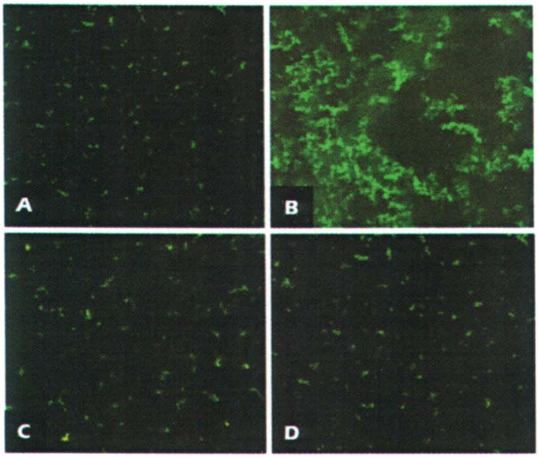

Figure 20.4. Confocal micrograph images of T_f-MAA-coated QDs (a) alone (b) 5 min after addition of anti-T_f mAb[12] Panels (c) and (d) show images of a thioglycerol-coated QD alone and 45 min after addition of antibody, respectively.

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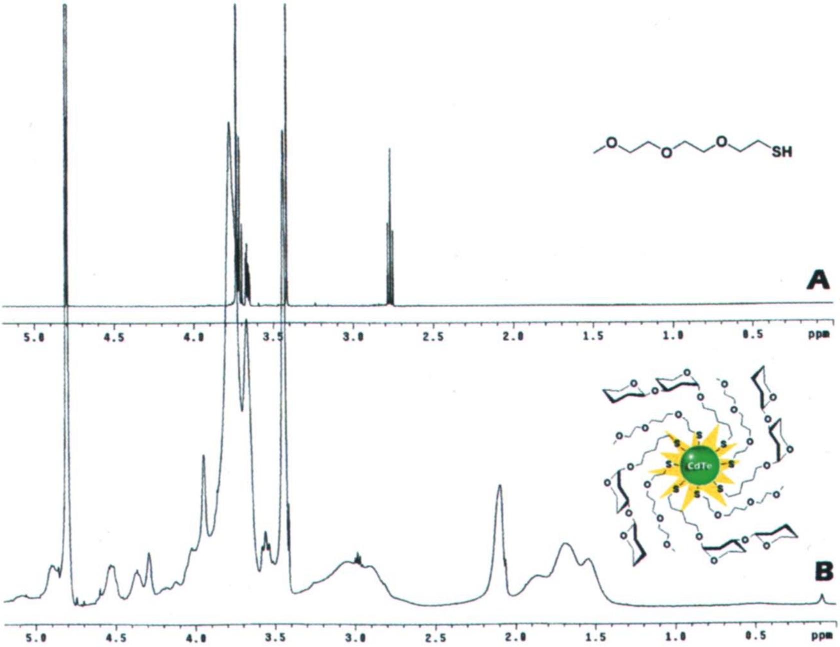

Figure 20.5. NMR spectra in D_2O of (a) free mercapto mTEG (mTEGSH) and (b) T_f-mTEG-CdTe hybrid QDs.

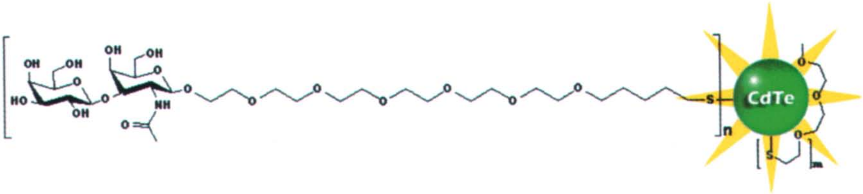

Figure 20.6. Structure of the optimum combination of linker and co-passivating agent for a biofunctional and robust T_f-coated QD.

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